APPROVED LABORATORY TECHNIC

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CLINICAL PATHOLOGICAL, BACTERIOLOGICAL, MYCOLOGICAL, PARASITOLOGICAL, SEROLOGICAL, BIOCHEMICAL AND HISTOLOGICAL

BY

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THIRD EDITION

Dedicated

TO THOSE

WHO HAVE ADVANCED THE SCIENCE OF CLINICAL PATHOLOGY

AND

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FOUNDER OF THE AMERICAN SOCIETY OF CLINICAL PATHOLOGISTS

PREFACE TO THIRD EDITION

New methods in laboratory diagnosis are developed yearly to meet the constant expansion of clinical pathology as a recognized specialty in medicine. Consequently the worker in this field must constantly keep abreast of newer methods developed by research, without forgetting or discarding those that are old and tried until they become obsolete and are replaced by those that are newer and better.

Among the new and approved methods included in this edition may be mentioned those of Quick for the quantitative determination of prothrombin; methods for the examination of semen, as kindly prepared for us by Dr. Werner Henle; Sparkman's method for the determination of urobilinogen in the urine and feces as a test for liver function as well as Quick's method for the estimation of hippuric acid in the urine for the same purpose; a method for the determination of serum lipase in relation to disease of the pancreas; the technic of the simplified Kolmer complement fixation test especially for use in laboratories required to conduct very large numbers of serum tests for syphilis, and the Eagle modification of the Wassermann test; methods for the determination of vitamin C in urine and plasma as now so frequently required; methods for the determination of sulfanilamide and other sulfonamide compounds in the blood and urine which have proven so valuable as guides in dosage in the treatment of disease, and finally a method for the determination of thiocyanates in the blood in relation to their administration in the treatment of hypertension. In addition many minor changes have been made with the correction of errors.

We are very grateful for the generous reception afforded the first and second editions and hope that this one will prove even more helpful to medical students, physicians, teachers, clinical pathologists and laboratory technicians. We are especially grateful to those who have so kindly favored us with numerous and valuable suggestions based upon years of experience as clinical pathologists and teachers. Our appreciation is also expressed to the publishers, D. Appleton-Century Company, for permission to enlarge the book in order to bring its contents strictly up to date, and for their unvarying courtesy and efficiency.

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PREFACE

IT is hoped that this manual will aid in the fulfillment of several of the objects of the American Society of Clinical Pathologists. namely, to establish standards for the performance of various laboratory examinations, to promote the practice of scientific medicine by a wider application of clinical laboratory methods to the diagnosis of disease and to encourage a closer coöperation between the practitioner and the clinical pathologist.

In the preparation of this edition, however, the authors have assumed responsibility for the selection of the methods given and therefore these can not be stated to have the approval of the Society as a whole. But the description of the technic of each method has been definitely approved by at least five members of the Society so that the title finally chosen for this edition of the book is "Approved Laboratory Technic, Prepared under the Auspices of The American Society of Clinical Pathologists."

In exercising a special effort to strike a proper balance in the amount of detail given to fulfill the needs of the laboratory technician without being burdensome to the expert, even the simplest details are frequently given with the hope that these will insure greater accuracy and uniformity in results and inspire greater confidence by physicians in laboratory examinations conducted by A. S. 'C. P. Approved Technic. And since there is a growing and gratifying increase in the use of laboratory methods by practicing veterinarians in the diagnosis of diseases of the lower animals, an effort has been made to render the manual of equal service to them.

It is realized that no amount of detail or simplicity of presentation can make up for the deficiencies of inexperienced. careless and incompetent technicians, but it is believed that the descriptions and illustrations are adequate for insuring accurate work by experienced and careful workers and for the teaching of clinical pathology.

Throughout the book an effort has been made to emphasize the importance of using accurate and reliable apparatus and reagents, as not infrequently the results of very careful and painstaking examinations and analyses are rendered worthless by inaccuracies in these particulars. For example, if blood counting pipets and counting chambers are inaccurately calibrated or broken. the counts can not be even approximately correct despite great care exercised in making them; if the antigen and other biological reagents employed in the complement-fixation test for syphilis are lacking in sensitiveness, the reactions can not be reliable regardless of the care and skill exercised in setting up the tests. Under such conditions any method may be very precise and the worker very careful,

but the results quite inaccurate and misleading. It is fitting and proper, therefore, to lay particular emphasis upon these and other sources of error.

Special emphasis has been placed upon quantitative tests and reactions, since these tend to greater accuracy and render more nearly possible approximately similar results from different laboratories. And in qualitative tests an effort has been made to suggest a uniform terminology and methods for reporting reactions.

Since laboratories are frequently required to secure specimens of blood, spinal fluid, gastric contents, bile, etc., for examination, methods for obtaining these are included.

While the field of "clinical pathology" is difficult to define, yet in practice it has come to include not only methods for the examination of blood, urine, feces, sputum, etc., but likewise those bacteriological, serological and chemical methods ordinarily requested in medical laboratories. Therefore, these subjects are included. Histological methods may be omitted and for this reason are not considered with the same amount of detail, although it is particularly gratifying to be able to include a chapter on Methods for the Microscopical Examination of Tissues by Dr. William C. MacCarty and Dr. W. L. A. Wellbrook.

The authors are particularly indebted to Mr. Alexander Keller, Jr., for assistance in reading and correcting the proofs of the section on Chemical Methods and for permission to use a number of methods from the Manual of the Biochemical Laboratories of the Graduate School of Medicine of the University of Pennsylvania, prepared mainly by himself with the coöperation of Dr. W. G. Karr and Dr. W. B. Rose under the direction of Professor George H. Meeker. Also, indebtedness to Mr. Herman Brown, Chemist to the Research Institute of Cutaneous Medicine, for several illustrations and assistance in preparing the section on Chemical Methods as well as to Dr. William G. Exton and Dr. Anton Rose, is acknowledged; also, to Dr. Elizabeth Yagle for assistance in preparing the section on Serological Methods and to Dr. Henry L. Bockus for assistance in preparing the chapters on the examination of stomach and duodenal contents and bile. And we especially beg to express deep appreciation of the unselfish, highly efficient, painstaking and prompt assistance rendered by the Committees of the Society whose names are gratefully given on the title page and without whose coöperation the book would not have been possible, as well as deep appreciation of the unvarying courtesy and efficiency of the publishers.

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GENERAL LABORATORY METHODS

CHAPTER I

THE MICROSCOPE AND METHODS OF MICROMETRY

EQUIPMENT

The microscope is one of the most essential and most frequently used instruments in the laboratory. If accurate observations are desired, the laboratory should be equipped with microscopes of recent manufacture by any one of the well-known companies in America. Bausch and Lomb, Spencer, Leitz and Zeiss offer instruments of the finest grade. There is no choice between them insofar as the optical systems are concerned but one may prefer one or the other type of stand design. For routine work the student model is to be preferred because of its simplicity and ruggedness. It is well to be familiar with stand and the names of its various parts. Consult the illustration in Figure 1. The microscope should be handled and carried only by the arm H.

The Optical System.—The optical system consists of (1) the objective which is the lens nearest the object above the stage. It is the chief lens and is compound i.e. made of a series of lenses. (2) The eyepiece or ocular which further magnifies the image formed by the objective. (3) The condenser which concentrates the light on the object as it rests upon the stage, thereby increasing the illumination. *Objectives* are named by their *equivalent* focal length. That is the distance between the principal focus and the optic center of a simple, single lens that would give an image of the same size as that formed by the objective at a distance of 250 millimeters. It means then that the 16 millimeter lens ($\frac{2}{3}$ inch) forms, at a distance of 250 millimeters, an image of the same size as a simple lens capable of causing parallel rays of light to come to a focus 16 mm. from its optical center. See Figure 2. This does not mean that the focal length of the objective or that its working distance (the distance from the object to the front of the lens) is 16 millimeters. Recently manufacturers have chosen to name lenses by their initial magnification which is more desirable. Bausch and Lomb achromatic objectives are designated as follows:

Magnification Number	Equivalent Focus in mm.	Working Distance in mm.
2	48	53.0
4	32	38.0
10	16	7.0
45	4	0.3
97	1.9	0.15

1
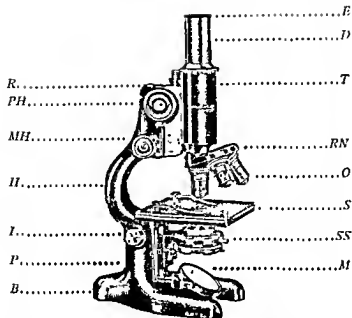
Fig. 1.—A Satisfactory Type of Microscope

E is the eyepiece, of which two or three are usually furnished for varying degrees of magnification.

D is the draw tube, which is calibrated and should always be drawn to 160 or any other length recommended by the manufacturer.

T is the body tube.

RN is the revolving or triple nosepiece carrying the objectives.

O is one of the usual three objectives.

R is the rack upon which the tube is raised or lowered.

PH is the pinion screw for coarse adjustment.

MH is the micrometer screw for fine adjustment.

H is the handle.

S is the stage.

SS is the substage carrying the Abbé condenser with diaphragm.

M is the mirror with plane and concave surfaces.

I is the inclination joint for using the microscope in an inclined position.

P is the pillar.

B is the base, which should be large and solid.

Fig. 2.—Focal Length of a Lens

X—X represents the optic axis. R, R, parallel rays of light brought to a focus at F. O represents the optic center. O—F is the focal length of the lens.

tion ...on is more helpful since oculars are also designated by their power and one need only multiply the initial magnification of the ... by that of the ocular to obtain the diam- of magnification of the optical system used, ...ovided the tube length is properly adjusted. Objectives are further named in accordance with their manner of use or construction.

Dry Objectives.—These are simple objectives, air alone lies between the front lens and the cover glass of the preparation.

Immersion Objectives.—Some are so constructed that water must be placed between the front lens and the cover glass for the objective to function properly. Others require more illumination than can be delivered by the condenser with air space or water between lens and objective. Cedar wood oil, which has the same refractive index as glass, must be placed between the front lens and the cover glass and between the glass slide and the condenser. These are the well-known oil immersion objectives. They will not permit full illumination or clear resolution without being immersed in oil. The common practice of using paraffin oil because it is less sticky is not recommended.

Achromatic Objectives.—These are objectives in which the image is free from the rainbow colors. A simple or single lens does not bring light of different wavelengths (different colors) to a common focal point. The light of shortest wavelength (the blue-violet end of the spectrum) is bent or refracted
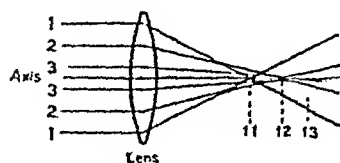
FIG. 3.—CHROMATIC ABERRATION WITH COMPOSITE LIGHT

White Light, a beam of white light composed of all the colors meeting a lens and the different wave lengths being differently refracted breaks the composite light up into its constituent colors.

Red Blue, the long waved red light is less refracted than the shorter waved blue light. After crossing at the foci the blue light is on the outside of the diverging cone, fb, fr. The focus of the blue light fb nearer the lens than the focus of the red light fr.

Axis, the optic axis of the lens. The dispersion or separation into colors differs with different transparent substances, and is not in proportion to the mean refraction. (From Gage, *The Microscope*, Comstock Pub. Co., Ithaca, N. Y.)

most by a lens and comes to a focus nearer the lens than light of a longer wavelength (the red end of the spectrum) which rays are bent or refracted less (Fig. 3). This means that the image of a colored object will not be in sharp focus for all colors. The phenomenon is called *chromatic aberration*. It may be corrected by placing a second, concave, flint glass lens behind the primary convex, crown glass lens. The use of fluorite or fluorspar in the second lens permits of a higher degree of correction. Achromatic objectives are corrected for two colors. They are also corrected at the same time for *spherical aberration*. It may be noted that a single hand lens gives an image in which the periphery is blurred or out of

FIG. 4.—SPHERICAL ABERRATION IN A LENS

Axis, The principal optical axis 1-2-3. Ray 1 at the edge comes to a focus at 11, ray 2 at 12 and ray 3 at 13. That is, the nearer the optic axis the longer the focus, and the nearer the edge of the lens the shorter the focus. (From Gage, *The Microscope*, Comstock Pub. Co., Ithaca, N. Y.)

focus, while the center is quite sharp. This is due to the fact that the periph rays of light are refracted or bent more than the more central rays and co quently come to a focus nearer the lens (Fig. 4). This may likewise be correct by placing a concave lens behind the primary convex lens. Correction is m with the same lens used for color correction.

Apochromatic objectives are corrected for spherical aberration and for thr colors and are, therefore, more desirable for photography. Aplanatic condensers are corrected for spherical aberration. They may also be achromatic.

Adjustable objectives are fitted with a small collar by which the distance be tween the front and back lens may be varied to compensate for variations in the thickness of cover glasses or mounting media. *Oculars* are usually designated by their magnifying power, as 5X, 7.5X, 10X, etc. It is their function to pick up the image formed by the objective and enlarge it further. Thus the degree of magnification of an object visualized may be determined by multiplying the magnification power of the ocular by the magnification number of the objective. (Tube length must be properly adjusted.) Oculars are also designated according to their construction. Huygenian oculars are the least expensive and those most commonly used. They do not possess the correction of better forms for color or flatness of field. Compensating oculars are overcorrected as to further reduce chromatic and spherical aberration of an objective. They should always be used in conjunction with apochromatic objectives.

Substage Condensers.—These are constructed usually of two lenses for the pur pose of concentrating light upon the object as it rests upon the stage of the microscope. They thereby increase the illumination. They are commonly of the nonachromatic type named after their designer Abbe. Aplanatic, achro matic condensers are available on the more expensive microscopes.
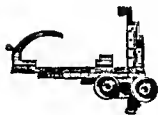
Fig. 5.—Mechanical Stage

The microscope should be equipped with two oculars preferably a 10X and 5X. Huygenian oculars are satisfactory if achromatic objectives are used. There should be three objectives on a triple, revolv ing nosepiece, namely a 16 mm. (E.F.), a 4 mm. and a 1.9 mm. (oil immersion). It is well to have available also a 32 mm. or 48 mm. very low power objective. Achromatic objectives are quite satisfactory for routine work. Compensating oculars and apochromatic or fluorite objectives must be used for microphotography. The microscope should be fitted with an Abbe condenser (aplanatic for photography). A mechanical stage is necessary for blood counting or wherever a systematic search of an object is to be made. The cheaper uncalibrated forms are quite satisfactory (Fig. 5). The monocular forms of micro scope are more commonly used because they cost less. The binocular microscopes are more desirable because they can be used with less fatigue over longer periods (Figs. 6 and 7).

Illumination.—Daylight from a north window is the ideal source of illumina-

tion. It is, however, so often unavailable that a more constant and dependable source is desired. Many forms of microscopic lamps are offered by manufacturers (see Figs. 8, 9 and 10). The substage lamp or any lamp utilizing a 100-watt nitrogen filled tungsten bulb and a "daylight" glass filter provides a satisfactory source of light. A powerful source such as the carbon arc or 6 volt ribbon filament bulb is required for dark-field illumination (*q.v.*)

Accessory Equipment.—It is well to be provided with a hand lens for the study of tissues (stained slides), sputa and feces, etc., prior to microscopic study. It gives
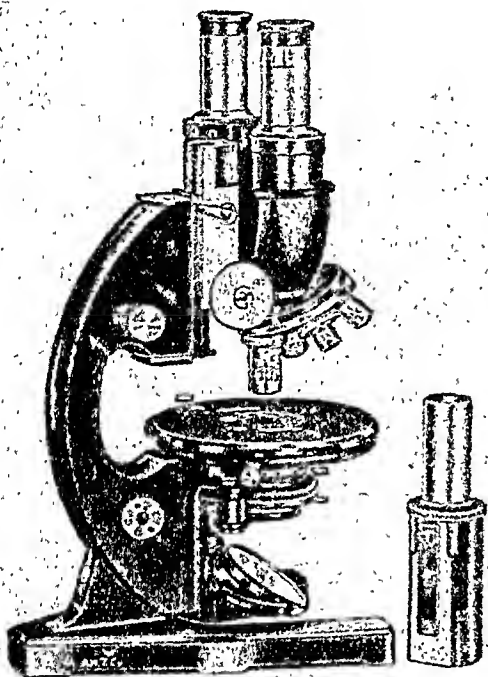
FIG. 6.—BINOCULAR MICROSCOPE   FIG. 7.—BINOCULAR TUBE ATTACHMENT
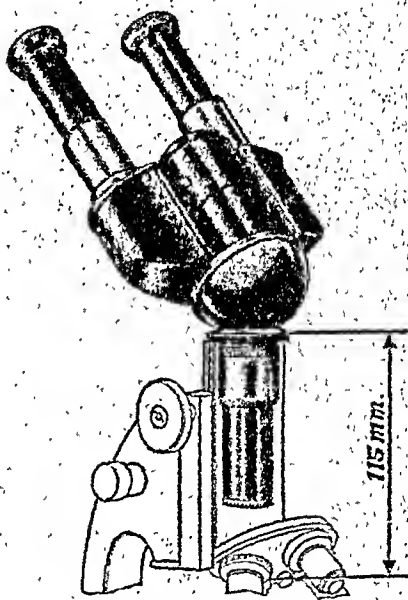
one an idea of the composition of the whole specimen and enables one to better orient himself when studying the smaller fields seen through the objective. In the selection of material from sputa or feces better samples will be obtained by the aid of such a lens. A pointer in the ocular is a great convenience. If the top of the lens of the ocular be removed, a diaphragm with a circular aperture will be found in the ocular tube. An eyelash or any fine hair may be so cemented on the rim of this diaphragm by means of Canada balsam so that the free end of the hair is in the center of the aperture. With the top lens in place the hair should appear as a fine pointer in focus. If it is not in focus move the diaphragm up or down until the hair is seen in sharp definition. This will serve to locate objects in the field. For the enumeration of small objects like blood platelets or reticulocytes it is helpful to cut down the size of the field. This may be accomplished by cutting a disk of black paper or metal to such a size that it will fit snugly over the diaphragm in the ocular. Cut a small square about 5 or 6 mm. on a side in the center of this disk. It can easily be removed when it is not needed.

Care of the Microscope.—The microscope should be covered at all times when not in use. A close mesh, lintless cloth boot that covers the entire stand is quite efficient. A glass bell jar or a transparent cellulose jar is convenient. If the glass jar is used, great care must be exercised in placing it over the microscope lest it strike and damage the instrument.

Fig. 8.—Micro Lamp, Chalet Form

Dust that accumulates should be wiped from the stand with a soft cloth kept for that purpose. The cloth moistened with xylol will remove cedar oil. Alcohol should never be used on any part of the microscope. The coarse adjustment screw and bearing should be cleaned from time to time as the lubricating oil tends to become thick and sticky. Remove the body tube from the stand, after racking it up as far as it will go, and wipe the bearing with a cloth moistened with xylol, until all dirt is removed. The parts should then be wiped with a clean cloth moistened with a good grade paraffin oil and the tube replaced. The bearings of the mechanical stage may be cleaned and lubricated in the same manner. The coarse adjustment screw may need tightening from time to time. The method differs with each make of microscope. Consult the book of instructions which comes with the microscope for directions. No other adjustments should be made except by an expert microscope mechanic. The agents for the manufacturer will gladly make such minor adjustments without charge. It is well to have the microscope inspected and adjusted occasionally by such an expert so that the life of the instrument will be prolonged and its efficiency maintained.

The lenses should be frequently brushed with a soft camel's-hair brush. Then they may be wiped and cleaned with a soft cloth (well washed linen) or lens paper. If the *cloth or paper is used before brushing, the* gritty particles always found in dust will scratch the lens surface. Oil may be removed from the immersion lens with a piece of lens paper moistened with xylol. The lens system must never be taken apart. The surface of front and back lens may be cleaned as directed above but never separated except by an expert microscope mechanic.
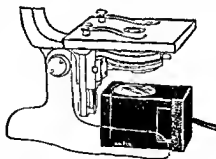
Fig. 9.—Micro Lamp, Substage Form

The Use of the Microscope.—The microscope should rest upon a table of such height that when one is seated before it one can comfortably look into the ocular without inclining the instrument. If seated before an open window or any

other source of light the eyes should be so shaded that no other rays of light enter the eye but those from the microscope. If using a monocular form, both eyes should be kept open and one should learn to relax the accommodation of the unused eye (Fig. 11). The ability to do this may be acquired by means of a black card. An opening is cut in a 3x6 inch card near the center of one narrow end, large enough to fit snugly over the upper end of the draw tube, after the ocular has been removed. The card then projects into the field of vision of the unused eye. If
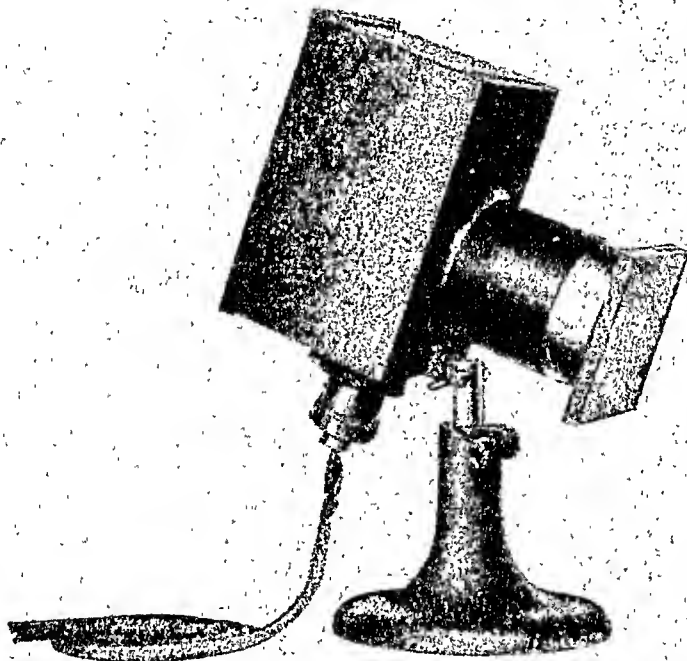
FIG. 10.—MICRO LAMP. FOR 100 WATT NITROGEN FILLED BULB OR RIBBON FILAMENT BULB (6 volt)

When equipped with the latter, the lamp is suitable for dark-field illumination (Bausch and Lomb).

the surface is blackened it reflects little or no light into that eye. After a time the card can be discarded.

The tube length should be adjusted. The draw tubes of most microscopes are graduated so that the tube may be pulled out to the proper length for each objective. The tube length for which each objective is corrected is engraved upon it. For most lenses this length is 160 mm. Tube length is extremely important with oil immersion objectives. A variation of 5 mm. will destroy the perfection of the image. Increased or diminished tube length alters the initial magnification of the objective. The tube should be withdrawn with a spiral motion while supporting the coarse adjustment screw lest the whole body tube be drawn from its bearing. It is returned to its former position with the same care lest the objective be driven forcibly against the stage or object. The microscope lamp should be placed about

10 or 12 inches from the mirror and its rays directed upon the mirror (plane surface). Swing the condenser out of position or rack it down to its lowest point. Look into the tube at the back of the low power (16 mm.) objective and manipulate the mirror with both hands until the tube is evenly illuminated. The ocular is now replaced and the object to be examined is placed upon the stage.

Focus the low power objective by first viewing it from the side and placing it down near (within one or two millimeters) the object. Then looking into the ocular rack the body tube upward by the coarse adjustment screw until the object

FIG. 11.—PROPER POSITION AT THE MICROSCOPE

Both eyes open. Body erect. Right hand manipulating the mechanical stage. Left hand manipulating the fine adjustment screw. (From Bass and Johns, Practical Clinical Laboratory Diagnosis, Waverly Press, Inc., Baltimore.)

comes into sharp focus. Illumination is now further adjusted by setting the condenser at the lowest point which gives even illumination yet brilliant sharp definition of the object. It is well to examine the objects always with the lower powers first as directed above, selecting fields to be studied with the higher magnification. Once the light is centered it need not be changed but the position of the condenser must be changed for each objective and for each variety of object studied. It must be higher for dense objects and quite low for unstained or transparent objects such as urinary casts. The illumination just described is called central illumination. It is the type most frequently used. After a little experience one may place objective

and condenser in their approximate positions and adjust the mirror without removing the ocular. The centering is finally determined by focusing up and down upon the object. If the illumination is correctly centered, the image moves up and down, in and out of focus. If not correctly centered the image will move to one side or another and back again as the objective is raised or lowered. Where the sharpest definition is desired, the aperture of the condenser must also be considered for each objective. After correct centering of the light and focusing of the condenser and objective, the ocular is removed and one looks at the back of the rear lens of the objective. Slowly the iris diaphragm of the condenser is closed until its shadow is seen in the periphery of the back lens.

With oil immersion objectives on bacteria the iris should be wide open. Where surface markings are to be studied, as on diatoms, cut the apparent aperture to about two-thirds and in still denser objects, as histologic preparations, to about one-half the opening of the back lens. With dry objectives the iris should be cut down to slightly less than the aperture of the objective so that its edges should just be visible in the periphery of the back of the lens.

With the oil immersion objective the condenser must be racked upward to its highest point. Oil should be placed between the condenser and the slide and between the cover glass and the objective. Focusing, with this and all objectives, should be *upward* after placing the lens near the object. *Never* focus down upon an object. The fine adjustment screw is used to study the object after it has been brought into focus by the coarse adjustment. The fine adjustment screw should never be turned more than one complete revolution in either direction. It is best operated by the left hand and constantly adjusted as the right hand manipulates the mechanical stage or the slide upon the stage.

In the study of surface contour, oblique illumination may be necessary. Research microscopes are so arranged that the iris diaphragm can be shifted from the center to the periphery of the condenser. Without this arrangement one must use a finger or a card over the aperture of the condenser so that the light may enter it from one side only. In this manner one may demonstrate the cylindrical shape of urinary casts.

Aside from these suggestions the adjustment of the condenser and the iris diaphragm become a matter of experiment in each case, in order to determine the best sort of illumination to bring out the desired details in the object one is preparing to study.

In order to obtain the best illumination for objectives more than 16 mm. E.F. the condenser should be removed and the plane or curved mirror used—depending upon the degree of illumination desired. When the condenser is used with objectives of 16 mm. E.F. or less (the higher powers) the plane mirror should always be employed. The condenser is so constructed that parallel rays of light are brought to a focus above the uppermost surface of the top lens of the condenser and in the plane of the object. If the concave mirror is employed, the rays of light are brought to a focus within the condenser and its effectiveness is depreciated. One may use the concave mirror and the condenser for the lower powers instead of removing

the condenser entirely and obtain satisfactory illumination of the field. Under these circumstances the condenser must be moved close to the object.

Coverglass Thickness.—A coverglass should be used with wet preparations. It is not so important when using the lower power objectives but even here the roundness of the drop formed causes a distorted field that is not so easily studied. A coverglass must be used with the higher power objectives lest the objective be wet by the liquid and the image be distorted and the lens of the objective be soiled. Covers are made of three standard thicknesses classified as No. 1, No. 2 and No. 3. From the optical standpoint No. 2 is best suited for all but oil immersion objectives. For these No. 1 is necessary because of the short working distance (space between objective and object). Even the best grades vary considerably in thickness and the variation affects the distinctness of the image. With the low powers this effect is so slight that it may be ignored but with the 4 mm. objective a deviation of 0.05 mm. above or below the standard for which the objective is corrected may obliterate fine structures in the image. One may avoid this difficulty by selecting covers of the proper thickness with a micrometer or by the use of the adjusting collar with which some objectives are fitted. The thickness of coverglasses is about as follows:

No. 1...............0.13 to 0.17 mm.

No. 2...............0.17 to 0.25 mm.

No. 3...............0.25 to 0.50 mm.

Slight variations in coverglass thickness may be compensated for by increasing the tube length for covers too thin or decreasing it for covers too thick. With a 4 mm. objective of 0.85 numerical aperture, an increase of 30 mm. in tube length will balance a decrease in coverglass thickness of 0.03 mm. It must be borne in mind that a change of tube length changes the initial magnification of the objective.

When employing oil immersion objectives, cover thickness is not so important, provided the combined thickness of it and the mounting medium is less than the working distance of the lens. Tube length is of considerable importance with immersion objectives, however, for a difference of 5 mm. is sufficient to destroy the perfection of the image.

Numerical Aperture.—It is well known that clearness of image, other things being equal, depends upon the width of the angle of light coming from the object; and that resolution of fine details depends largely upon the width of the angle of such rays that can enter the objective and become effective in producing the microscopic image (angular aperture). Angular aperture may be defined as the angle formed by the border rays of light passing from the object into the front lens of the objective (Fig. 12). Were it not for the use of immersion substances as oil and water, this angle would be a measure of the resolving power of a lens. Resolving power is the property by virtue of which a lens is capable of showing distinctly two tiny bodies lying side by side. The greater the resolving power, the smaller and closer may these bodies be. By the use of immersion substances wider

angular apertures are obtainable than with air and these require the consideration of the refractive index of the substance used. Abbe suggested the term numerical aperture which he obtained by the product of the lowest refractive index of material between the object and the front lens of the objective (air for dry objectives,
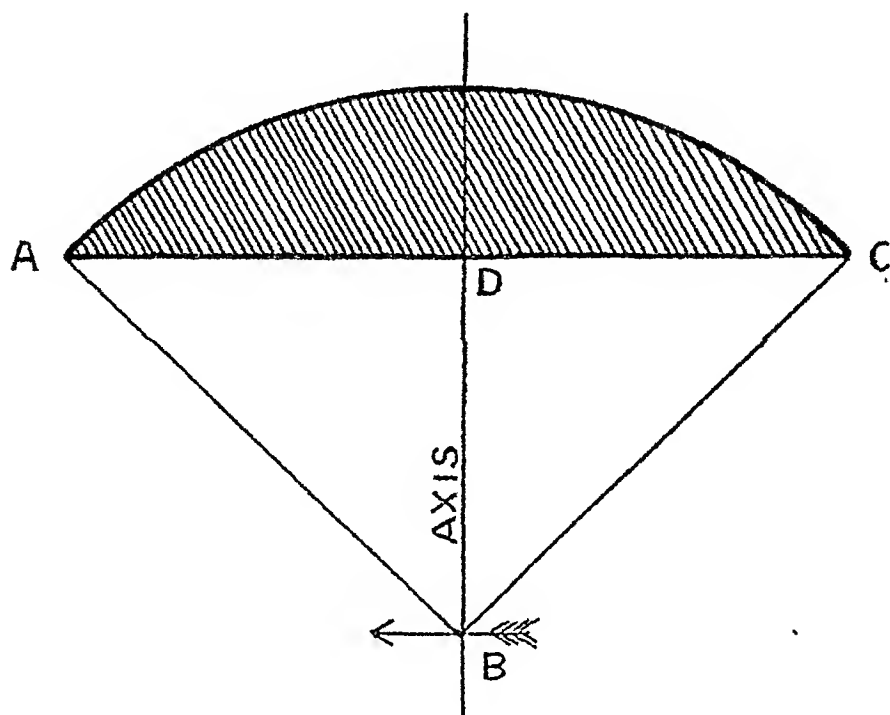
Fig. 12.—Angular Aperture of an Objective

(AXIS), The principal optic axis of the objective; (B) The object just outside the principal focus; (ADC) Diameter of the front of the objective and the base of the angle of aperture; (ABD) Half the angle of aperture (u); $\frac{AD}{AB}$ Represents the sine of u." (From Gage, *The Microscope.* Comstock Pub. Co., Ithaca, N. Y.)

water for water immersion and cedar oil for homogeneous immersion) and the sine of half the angle of aperture of the objective. The formula is as follows:

$$\text{N. A.} = n \sin u$$

n—the lowest refractive index that appears between the object and the front lens of the objective

u—Half the angular aperture of the objective

N. A. is always engraved upon the objectives

It is important that condensers be used which can produce illumination of sufficient N. A. to fill the objective. There is no point in employing a costly objective of N. A. 1.40 with a condenser of N. A. 1.00 or 1.25. If the full angle is to be obtained, both condenser and objective must be immersed in oil.

Increase in N. A. renders correction for spherical and chromatic aberration more difficult and reduces the depth of focus of the objective. Objectives of high numerical aperture produce images that are extremely sensitive to changes in the fine adjustment screw.

MICROMETRY

A good micrometer is a practical necessity, as the importance of size in identification of microscopic structures cannot be too strongly emphasized. Even very rough measurements will often prevent humiliating blunders. The principal microscopic objects which are measured clinically are bacteria, animal parasites and their ova, and blood corpuscles. The metric system is used almost exclusively. For very small objects, 0.001 millimeter has been adopted as the unit of measurement, under the name micron. It is represented by the Greek letter μ. For larger objects,

where exact measurement is not essential, the diameter of a red blood corpuscle (7 to 8 micra) is sometimes taken as a unit. Of the several methods of measurement, the most convenient and accurate is the use of a micrometer eyepiece (Fig. 13). In its simplest form this is similar to an ordinary eyepiece, but it has within it a glass disk upon which is ruled a graduated scale. When this eyepiece is placed in the tube of the microscope, the ruled lines

Fig. 13.—Micrometer Eyepiece with Movable Scale
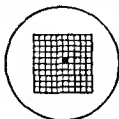
Fig. 14.—Ocular Micrometer Disk

appear in the microscopic field, and the size of an object is readily determined in *terms of the divisions of this scale.* The value of these divisions in millimeters manifestly varies with different magnifications. Their value must, therefore, be determined separately for each objective. This is accomplished through use of a stage micrometer—a glass slide with carefully ruled scale divided into subdivisions, usually hundredths of a millimeter. The stage micrometer is placed upon the stage of the microscope and brought into focus. The tube of the microscope is then pushed in or pulled out until two lines of the one scale exactly coincide with two lines of the other. From the number of divisions of the eyepiece scale, which then correspond to each division of the stage micrometer, the value of the former in micra or in fractions of a millimeter is easily calculated. *This value, of course, holds good only for the objective and the tube length with which it was found.* The counting slide of the hematocytometer will answer in place of a stage micrometer, the lines which form the sides of the small squares used in counting red blood corpuscles being 50 micra apart. When using the counting chamber with an oil-immersion lens a cover must be used, otherwise the oil will fill the ruled lines and cause them to disappear. Any eyepiece can be converted into a micrometer eyepiece by placing a micrometer disk—a small circular glass plate with ruled scale—ruled side down upon its diaphragm (Fig. 14). If the lines upon this are at all hazy the disk has probably

been inserted upside down or else the diaphragm is out of its proper position. Usually it can be pushed up or down as required.

DARK-FIELD MICROSCOPY

In the preceding pages, bright field microscopy has been discussed wherein objects have been studied microscopically by transmitted light, that is to say, light from the condenser passed through the object and was modified by the natural color or artificial stain of the object. Those objects which possess little or no differential coloring of their various parts are stained by selective dyes to cause them to stand out prominently. Thus the nuclei of cells are rendered visible in the cytoplasm, and bacteria barely visible are made quite prominent. Often it is desirable to study living things that cannot be easily stained or would be destroyed by a dye and are yet so tiny that even with the oil immersion lens they would be invisible by transmitted light.

FIG. 15.—DARK-FIELD CONDENSER

As one gazes about the ordinary room no dust particles are seen by the naked eye, but, if one directs the line of vision at right angles to a beam of sunlight, entering through a window, myriads of tiny particles become apparent because they reflect the rays of sunlight into the eye. One sees them not because the light is brighter but because they reflect the sun's rays into the eye. The phenomenon is spoken of as the "Tyndall effect." By the same principle, nonluminous stars or the moon are seen in the sky at night because

FIG. 16.—SECTIONAL VIEW OF PARABOLOID DARK-FIELD CONDENSER

of the sun's rays which they reflect to the earth. This is the principle of dark-field illumination. No light enters the microscope except that reflected by the objects in the field. Hence, the background is dark and the objects appear bright. Illumination may be from above the stage or from below it. When illuminated from below the effect may be obtained with the lower power objectives by placing a metal disk supplied with many microscopes beneath the iris diaphragm of the Abbe condenser. This shuts out the central rays and allows only the peripheral border rays to enter.

When higher magnification is required as in searching for *Treponema pallidum*, greater obliquity of light is necessary because of the increased numerical aperture of these objectives. One of the regularly designed dark-field illuminators must be employed (Figs. 15, 16, 17). One may use one of the combined types. These are refracting condensers of the Abbe type fitted with interchangeable lens mounts. The advantage of such a condenser is that one may shift from bright field illumination to dark-field by lowering the condenser and changing the top element. This type is not a very satisfactory dark-field illuminator. The paraboloid illuminator is the most practical for routine work (see Fig. 18). It is easily manipulated

and gives an excellent dark field. The cardioid illuminator provides light rays of even greater obliquity and, therefore, can be used with objectives of higher numerical aperture with all of its advantages. The result is a brighter object with a much darker background and greater resolving power. (Fig. 19.) However, the cardioid illuminator is much more difficult to manipulate, and it is much more sensitive to dust particles or scratches upon the slide or enverglass. The paraboloid type is recommended and the method of using it described as follows:

1. The oil-immersion lens has too great a numerical aperture for this work. It must be cut down by placing a funnel stop behind the rear lens. The proper stop is furnished by the maker of the objective. It should provide a numerical aperture of 0.80. The threaded end of the objective is unscrewed and the stop placed in position, apex down in the lens mount. The threaded end is then screwed back in place and the objective is screwed into the nosepiece.

2. The Abbe condenser is removed from its substage adjusting sleeve and the dark-field illuminator fastened in its place. One must make certain that the upper lens surface is in the plane of the upper surface of the stage or a little higher when the illuminator is racked to its highest point. Close contact with the under surface of the glass slide may then be assured. The diaphragm of the illuminator should be wide open.

3. The source of illumination, a carbon arc lamp or a ribbon filament lamp should be placed about 12 inches from the mirror and a narrow beam of light directed on the plane mirror. The beam should nearly fill the mirror surface.

4. As the light from the lamp is reflected up into the illuminator, some of it is reflected by the polished under surface of the illuminator back into the mirror. This light may be reflected on the wall behind the lamp or on the lamp housing as a very much dimmer light spot than that which comes

FIG. 17.—A DARK-FIELD CONDENSER WITH LIGHT ATTACHED
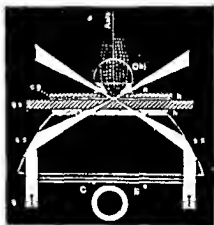
FIG. 18.—PARABOLOID CONDENSER

The drawing shows the path of light through the condenser. C.S. is the central stop. S.S. the silvered parabolic surface; H.H. homogeneous liquid between the condenser and slip. Canada balsam inclosing the object and the homogeneous liquid between the object and the cover glass; G.S. Glass slide on which the object is mounted. It must be of a thickness to bring the focus of the cone of light on the object; C.G. cover glass; Obj. front lens of the objective. (From Gage, *The Microscope*, Comstock Pub. Co., Ithaca, N. Y.)

from the light source directly. These two spots of light may be easily seen if the mirror is manipulated. The mirror will be correctly placed if the dim spot reflected from the illuminator can be directed back into the light source through the front lens of the lamp. The low power objective is focused on the upper surface of the condenser before placing the preparation on the stage. When some light passes into

the illuminator even though the illumination is not uniform, a small circle will be seen, scratched upon the surface of the top lens. This indicates the center of the lens and by means of the centering screws on the illuminator mount, this small circle must be brought into the exact center of the field.

5. One is now ready to prepare the material for examination. Glass slides of a definite thickness must be selected. The correct thickness is usually indicated upon the illuminator mount. The Bausch and Lomb illuminator is adjusted for slides of not greater than 1.55 mm. If thicker slides are used, the illuminator cannot be brought into proper relation with the object. If the slide is too thin, the illuminator may be racked down to the proper level. The coverglass must not be more than 0.18 mm. thick (No. 1). The material to be examined must be in a thin emulsion. Too many particles will cause too much scattering of light and diminish the sharpness of the field. The scraping or juice expressed from chancres' in searching for treponema pallidum must not contain too much debris. pus or blood. It is well to practice making suspensions of varying density with salt solution. Scrapings from the margins

FIG. 19.—DIAGRAM OF A CARDIOID CONDENSER WITH URANIUM GLASS IN HOMOGENOUS CONTACT WITH ITS UPPER FACE TO SHOW THE COURSE OF THE HOLLOW CONE OF LIGHT

C.S, Central stop; U. block of uranium glass whose fluorescence marks out the path of the light cone; Si, Ci. ground-glass slip much too thick showing the lighted circle and dark center above the focus; S2. C2, ground-glass slip of the correct thickness to show the focus of the condenser at its ground surface; S3, C3, S4, C4. ground-glass slips equally too thick and too thin to show a similar bright circle and dark center above and below the focus. (From Gage, The Microscope, Comstock Pub. Co., Ithaca, N. Y.)

of the gums will provide excellent material with which to practice.

6. Raise the illuminator so that its upper surface is in the same plane as the surface of the stage. Place a drop of immersion oil upon the illuminator lens and place the preparation in the oil in position. Examine the preparation with the 16 mm. objective. If the condenser is in proper position, a circle of light will appear in the center of the field. This spot is more easily seen if ground glass slide of exactly the same thickness as the one used for the preparation is substituted, ground side placed upwards (Fig. 20). (The ground glass slide which should be a part of the equipment can be made by rubbing a slide of proper thickness in a paste made of water and very fine emery or carborundum powder on a piece of plate glass.) If the illuminator is too high or too low, a ring of light with a dark center will be seen. Raise or lower the illuminator until a circle is obtained. If this

is not possible, the slide is too thick or the illuminator is not properly set in its holder. If the illumination appears to be correct, swing the immersion lens into position and after placing a drop of immersion oil upon the cover bring the objective into focus (focus upward). The background should be dark and the
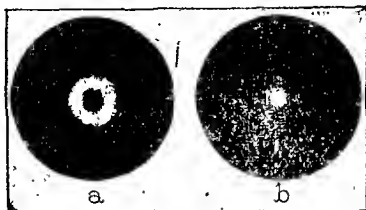
FIG. 20.—DARK-FIELD ILLUMINATION

(*a*) Improper illumination because the condenser is above or below the correct focus. (*b*) Proper illumination with the bright spot when the condenser is correctly focused. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

scattered bodies in the field should be brilliantly illuminated. Slight adjustments of the height of the condenser and the position of the mirror will improve the blackness of the background and the brilliance of the illuminated bodies. Finally, when the best setting is obtained slowly close the iris diaphragm until there appears the most contrast of brightness of object and blackness of field. As in the bright field microscope exact centering of the illuminator will be evidenced by an up and down movement of the image as the fine adjustment screw raises or lowers the objective in and out of focus. A lateral motion of the objects in the field indicates that the illuminator is not centered.

Sources of failure in dark-field illumination:

(*a*) Insufficient illumination
(*b*) Condenser out of focus or decentered
(*c*) Glass slide too thick
(*d*) Failure to close iris diaphragm or to use funnel stop of correct N. A.
(*e*) Preparation too dense

CHAPTER II

METHODS FOR HOUSING, FEEDING, INOCULATING, BLEEDING AND AUTOPSYING ANIMALS, AND DIAGNOSIS OF ANIMAL DISEASES

Principles.—1. Suitable animals are indispensable for certain laboratory procedures, as follows:

(*a*) For the detection of tubercle bacilli in sputum, urine and other materials; for the detection of tetanus and other anaerobic bacilli in wound discharges; for the detection of glanders, anthrax, tularemia and other organisms in various discharges and materials; for the detection of *Spirochaeta pallida* in spinal fluid and various tissues; for securing a rapid growth of pneumococci for type differentiation in relation to the serum treatment of pneumonia; for testing the virulence of diphtheria bacilli in relation to the lifting of quarantine in diphtheria and for raising the virulence of some pathogenic organisms, notably streptococci and pneumococci.

(*b*) For aiding the isolation of certain organisms, as tubercle and glanders bacilli, pneumococci, etc.

(*c*) For the preparation of vaccines and the propagation of certain viruses that cannot be cultivated artificially, as in smallpox and rabies.

(*d*) For preparing sera for diagnostic immunological reactions like complement-fixation agglutinins, precipitins and various hemolysins.

(*e*) For testing the curative activity of various chemical agents like arsphenamine and other organic arsenicals employed in the treatment of syphilis and other diseases.

(*f*) For the preparation of various immune sera employed in the prophylaxis and treatment of diphtheria, tetanus, meningococcus meningitis, gangrene, pneumonia, etc.

(*g*) For testing the antibody strength of diphtheria, tetanus, pneumococcus and other immune sera.

2. Animals should be healthy, selected with care, and provided with comfortable and clean housing as well as sufficient and appropriate food.

3. Few of the methods of injection and bleeding produce any more pain than similar procedures in human beings, but all major operative procedures should be conducted under full anesthesia and all animals at all times should be handled and treated with the tender care that their great service to humanity, as well as to the lower animals themselves, well merits.

17

HOUSING ANIMALS

1. The cages for small animals such as rabbits, guinea-pigs, mice and rats should be so constructed and arranged that the animals are kept dry, comfortable and sanitary. Avoid overcrowding. Separate cages should be provided for normal unused animals.

2. Animals from outside sources should be carefully inspected for evidence of disease before being placed in the normal cages. If space
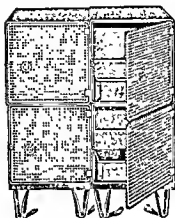
Fig. 21.—Animal Cage
(Lewis)
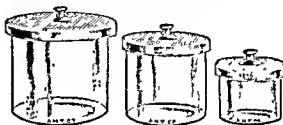
Fig. 22.—Animal Jars for Mice, Rats and
Guinea-Pigs

permits, the new animals should be quarantined for a week or ten days.

3. The cages should be constructed to permit thorough cleansing and disinfection
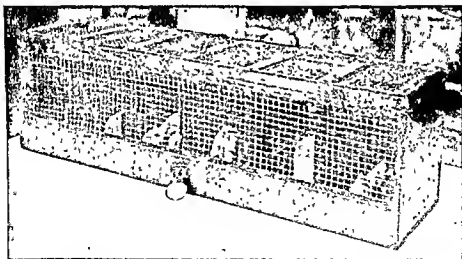
Fig. 23.—A Cage for Keeping Rats and Mice Separately
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

and arranged for sufficient light and ventilation. Various models are available from supply houses (Figs. 21 and 22) or may be built to meet special requirements.

4. Special isolated quarters or cages should be provided for animals inoculated

with very infectious material and especially that known or suspected of containing *B. anthracis, B. mallei (Pfeifferella mallei), B. tularense (Pasteurella tularensis),* rabies, hog cholera, foot and mouth disease, etc.

5. Inoculated animals should be kept alone in cages to guard them from being annoyed and even injured by normal animals (Fig. 23).

6. The animal quarters should be well lighted and ventilated. A uniform temperature during the entire day and night should be provided.

IDENTIFICATION OF ANIMALS

A dependable system of identification of the animals should be adopted. The practice of cage labeling is suitable for stock animals kept in large groups, although in the interest of complete records each animal should be numbered and registered with regard to sex, description, etc.

1. **Tagging.**—For rabbits and guinea-pigs a small aluminum tag may be used. It is held in place by small staples passed through the eyelets of the tag and then through the ear and the ends bent over (Fig. 24).

For larger animals as horses, cows, pigs, etc., there are various types of tags which are supplied with special instruments for attaching. These can be obtained through veterinary supply houses.

2. **Banding.**—This method of identification is chiefly employed for fowl. A band bearing the number is fastened around the leg. There are several types, commonly called "leg bands," which can be purchased from poultry supply houses.

3. **Description.**—If this method of identification is employed alone, animals of different markings or color should always be selected if more than one is to be placed in a cage. Rubber stamp

FIG. 24.—ANIMAL TAGS

drawings of the mouse, rat, guinea-pig and rabbit may be purchased to facilitate these records.

This method is usually used in conjunction with the tagging method to insure proper identification should the tag be lost.

Any deformities or peculiar markings should also be carefully noted.

The sex should be recorded as a part of the description (male ♂ and female ♀).

4. **Marking.**—Small animals like mice and rats may be marked by coloring the hair and skin with dyes (saturated alcoholic solutions of fuchsin or picric acid) on the body or along the tail. Cages should be labeled.
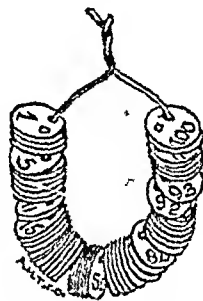
FEEDING OF ANIMALS [1]

1. **Mice.**—Mice may receive stale bread soaked in water or skimmed milk. A small amount of cod liver oil may be added once a week (approximately one ounce for 300 mice). Crushed barley or rolled oats or moistened middling may be offered at regular intervals. An excellent ration recommended by Keeler consists of rolled oats (240 parts), powdered skim milk (30 parts), cod liver oil (8 parts),

[1] K. F. Meyer, *J. Lab. & Clin. M.* 1932, 17:510.

and salt (one part). The formula for rat feed prepared by Maynard (*Science* 71:192, 1930) is equally satisfactory. Greens in the form of lettuce or clover should be given occasionally. Drinking water must be always available.

2. **Rats.**—One can either use the Maynard standard diet or prepare a mash which consists of *boiled beans, wheat maize meal, cabbage, and cod liver oil. Beef or liver and fresh cabbage* should be offered once a week. A mixture of boiled vegetables supplemented by oats, corn, or white bread mixed with milk is equally satisfactory. Drinking water should always be available.

3. **Guinea-pigs and Rabbits.**—Rolled oats or crushed barley, bran, and a good quality of alfalfa or clover hay represent the basic ration which must be supplemented with green feed, either cabbage or carrots, and beets. From time to time, salt, fish meal, and boiled potatoes may be offered. If the food contains considerable moisture, no water need be supplied. Spontaneous scurvy is by no means uncommon among guinea-pigs maintained on a diet which lacks green stuffs.

Aside from a careful selection of freshly prepared food mixtures, or wholesome vegetables and greens freed from tainted or rotten spots, it is important that a regular system of feeding be strictly adhered to. All animals should be fed and cared for once a day, including Sunday. A double ration of food thrown into the cages on Saturday will not supply the required nourishment on Sunday.

CLINICAL OBSERVATIONS OF INOCULATED ANIMALS

1. Inspection once or preferably twice daily for *general appearance* should be given with a view to detect symptoms. Each animal should be observed in motion; the consumption of food and water and the appearance of the feces should be noted. Attention should also be paid to possible salivation, nasal and conjunctival discharges, and to the reactions at the site of inoculation.

TABLE I

Normal Temperature, Pulse, and Respiration of Experimental Animals

Animal	Average Rectal Temperature °C. and °F.	Pulse Rate	Respiration Per Minute
Guinea-pig	38.6° C.; 39.4° C.—7.5 cm. from anus (101.5° F.—4 cm. from anus) (Minimum 37.8° C., maximum 40.5° C.)	150	100 150
Rabbit	39.6° C. (102.4° F.) (Minimum 38.3° C., maximum 40 8° C. No temperature under 40 0° C. should be considered pathologic)	120 140	50 60
Rat	37.9° C. (100 0° F.)	—	210
Mouse	37.4° C. (99.3° F.)	120	—

2. The *weight* should be recorded before experiment and afterwards at weekly intervals or more often as the circumstances require. Each weighing should be done as nearly as possible under the same conditions as the first one.

3. The *temperature* should be taken in many cases before beginning the particular experiment and subsequently on each successive day at the same hour. For the sake of convenient reference the normal average temperature is given in Table I in order to prevent the erroneous assumption that a pyrexia is present in an animal which shows merely its own normal temperature.

4. A *hematologic study* is frequently indicated. The general principles are the same as customarily used in the diagnostic laboratory. The normal averages of the different blood cells are summarized on page 62.

5. *The bacteriologic, serologic, and autopsy* examinations differ in no way from those generally employed.

SELECTION OF ANIMALS FOR DIAGNOSTIC TESTS

1. **Staphylococcus Infections.**—The pathogenicity of a staphylococcus should be studied on the rabbit or on Japanese white mice. Significant lesions may be observed following intravenous injections, while local reactions may be induced by scarification of the cornea. Toxic metabolic products may be tested by intravenous injection or by intradermal application.

2. **Streptococcus Infections.**—There is some difference of opinion as to which animal is most susceptible. If relative sizes of animals are considered, the rabbit is the most readily attacked. Various methods of infection are chosen in order to imitate the pathologic process from which the coccus has been isolated. Virulence tests are frequently made on mice. Great care should be exercised in the interpretations of the bacteriologic findings on these animals since several workers have found that hemolytic and nonhemolytic streptococci have been isolated from supposedly "normal mice." Therefore, the supposed mutation of pneumococci and nonhemolytic varieties by intraperitoneal injections deserves further investigation. Latent infections due to hemolytic streptococci are quite common in guinea-pigs. They frequently invalidate the diagnostic experiments.

3. **Pneumococcus Infections.**—The extreme susceptibility of the mouse to the pneumococcus is a commonplace of laboratory experience and forms the basis for the rapid isolation of the organism from bacterial mixtures, sputum, etc. Although the mouse may be killed by a smaller dose than the rabbit, it is generally recognized that the rabbit is more susceptible. In an emergency this animal can therefore be used for the typing of pneumococcus strains. Experimentation on guinea-pigs requires careful and critical interpretations since spontaneous pneumococcal infections may be unusually common in the stock of certain breeders.

4. **Influenza Bacilli.**—It is well to remember that autolyzed influenza bacilli may set up spontaneous streptococcal or pneumococcal infection in guinea-pigs and mice. A general blood infection may be produced regularly when the sputum to be tested contains the symbiotic adjuvants, the cocci.

5. **Tuberculosis.**—The detection of tubercle bacilli by animal inoculation offers several disadvantages. Not infrequently these animals suffer from chronic diseases, such as pseudotuberculosis or Salmonella infections which present gross anatomic lesions readily confused with those of tuberculosis. Spontaneous tuberculous infec-

tions of the guinea-pig and rabbit have been reported by many workers and thus may greatly invalidate the significance of the test. In part, these disadvantages may be overcome by a judicious interpretation of the postmortem findings. It is a well-known fact that in susceptible animals the primary localization of the lesion always indicates the avenue of infection. If, for example, after subcutaneous inoculation a tuberculosis of the tracheobronchial lymph nodes with no disease in the inguinal nodes is found, one may conclude that the infection was acquired from extraneous sources.

6. **Plague.**—*B. pseudotuberculosis rodentium* (Pfeiffer) occurs spontaneously in guinea-pigs and may thus interfere with the diagnosis of plague.

7. **Brucella Infections.**—It is not generally appreciated that isolated cases of melitensis and abortus infections have been reported in animals. An extensive epizootic which was caused by a melitensis type and affected 400 guinea-pigs has been reported by Zdrodowski. In view of the wide distribution of the Brucella organisms, it is imperative that in the future shipments of guinea-pigs from unknown breeders be scrutinized for Brucella infection. Agglutination tests previous to the inoculation of the test material are exceedingly useful.

8. **Anaerobic Infections.**—Those who use guinea-pigs or rabbits for the primary isolation of a pathogenic anaerobe should always remember that certain species may be found as common parasites in the intestinal canal or even in the organs of these animals.

9. **Paratyphoid Infections.**—The fact that *B. aertrycke* orally administered to mice sets up a lethal septicemia, while *B. paratyphosis B* (Schotmüller) usually does not, is sometimes used as a practical differential test of these two organisms. Unfortunately, its value is greatly reduced on account of the widespread occurrence of latent aertrycke infection in rodents. Experimentation with paratyphoid bacilli on mice requires sound judgment. The bacteriologic literature reports such bizarre findings as the transformation of a *B. para-typhosus* into a *B. enteritidis* or *B. aertrycke.*

10. **Virus Infections.**—The controversial literature on experimental encephalitis clearly shows that latent widespread parasitic infections may be responsible for misleading conclusions. Furthermore, the discovery of a new virus by Miller, Andrewes and Swift, in the course of an attempt to reproduce experimental rheumatic fever, furnishes another example of the many obstacles which may continue to render the animal test a diagnostic procedure having considerable complications.

Many more observations could be cited. In particular, attention might be drawn to the deficiency of the hemolytic complement in the blood of certain races of guinea-pigs owing to the absence of the third component (Hyde). Suffice to emphasize that the bacteriologist who employs the animal as a test object or model in his diagnostic work should be in a position to defend his claims that the lesions or findings are not those of a spontaneous disease but the result of the experiment. This aim can be met to a great extent if he employs only well-bred animals with a known hereditary history free from bacterial infections and parasitic

invasions. maintained and cared for by an experienced personnel, and kept on well-balanced diets in a sanitary and hygienically controlled environment.

DISEASES OF GUINEA-PIGS

1. **Pneumonia.**—The causative organism in most cases is a hemolytic streptococcus. Occasionally sporadic cases are due to infection with the pneumococcus. The *B. bronchisepticus* (*Alcaligenes bronchisepticus*) and Friedländer's bacillus (*Encapsulatus pneumoniae*) have been reported as producing pneumonia in these animals. The symptoms are loss of appetite, roughened coat, and rapid breathing.

Autopsy findings are congestion with consolidation of lungs, which may be associated with pleural and pericardial exudates of a serous or fibrinous character.

2. **Abscesses.**—Abscesses result from an infection of the lymph glands with *hemolytic streptococci*. They often become very large and are usually encapsulated. The contents are a thick and creamy pus from which streptococci can be isolated.

They may become enzootic or even cause epizootics among guinea-pigs. This condition is often called "epizootic lymphadenitis," and the lymph nodes of the neck and axilla are most commonly infected. It is not usually fatal unless the abscesses become so large as to interfere with the function of important organs.

After rupture and drainage the animal usually recovers. It is advisable to incise, remove the pus, and allow good drainage.

3. **Paratyphoid.**—This disease is caused by bacteria belonging to the Salmonella group. The organisms usually encountered are closely related to the *B. aertrycke* or the *B. enteritidis*.

The symptoms are loss of appetite, roughened fur, emaciation and weakness. In acute cases death often occurs before symptoms are noted. Outbreaks among laboratory animals may cause considerable loss. The mortality varies from 40 to 70 per cent.

At autopsy, the spleen and liver show the most constant changes. The former is enlarged and soft. studded with small foci or large yellowish nodules and often covered with a plastic exudate. The liver shows small necrotic foci. The intestines are injected and show swollen Peyer's patches. Pleurisy, pneumonia and purulent endometritis may be present.

4. **Pseudotuberculosis.**—This disease is characterized by the formation of whitish nodules in the liver and spleen associated with enlarged lymph glands which often become abscessed. The causative organism is the *B. pseudotuberculosis* (*rodentium*), which is a small, coccoid, gram-negative, nonmotile and distinctly bipolar organism. It will grow an ordinary culture media under aerobic conditions. It is difficult to demonstrate in chronic lesions. It can be definitely identified by an agglutination test with specific serum.

Three clinical types are recognized: (*a*) septicemic type (death in one or two days): (*b*) emaciation and diarrhea (death in three to four weeks), and (*c*) glandular type.

The diagnosis is made by bacteriological examination and the characteristic lesions.

Control measures are not recommended, because of lack of knowledge concerning the epidemiology of the disease.

5. **Tuberculosis.**—Natural infections of guinea-pigs with tubercle bacilli are not common. Normal guinea-pigs can readily contract the disease from tuberculous cage mates or even from animals in the same room.

DISEASES OF RABBITS

1. **Nasal Catarrh (Snuffles).**—This condition may be caused by bacterial infection or by coccidia. When due to bacteria it is spoken of as "snuffles" and when due to coccidia (*Eimeria stiedae*) as "nasal coccidiosis."

The organisms most commonly found are *B. lepisepticus* (*Pasteurella cuniculicida*) and *B. bronchisepticus* (*Alcaligenes bronchisepticus*). The latter when present is usually associated with the former.

The diagnosis of nasal coccidiosis is made by finding the coccidia in the nasal secretions.

The symptoms are sneezing, nasal discharge which is recognized by wetting of the hair around the nasal openings, or the presence of mucus and emaciation. Inflammation of the eyes is often present in cases due to coccidia. In the bacterial type the infection may extend to the lungs and cause pneumonia.

2. **Pneumonia.**—This is usually the result of extension of infection from the upper respiratory tract. The organisms usually found are the *B. lepisepticus* and the *B. bronchisepticus* (*Alcaligenes bronchisepticus*).

3. **Coccidiosis.**—This disease is caused by coccidia (*Eimeria stiedae* and *Eimerio perforans*), which are among the commonest parasites found in the rabbit. Although they are found in the intestinal contents of apparently normal rabbits, they are capable of invading the epithelium and causing a mild catarrhal to a severe type of enteritis (intestinal coccidiosis).

They commonly invade the epithelium of the bile ducts, gallbladder and liver. Multiple small white nodules result from their invasion of the liver (hepatic coccidiosis).

The nasal form of the disease has been given above, under nasal catarrh of rabbits (snuffles).

The diagnosis is made by finding the coccidia in association with enteritis or nodules. The organism is readily demonstrated by placing the contents of a liver nodule, bile, or scrapings of intestinal mucosa on a slide and examining the unstained specimen.

The oöcysts appear as oval bodies from 15 to 25 micra in length.

There is no treatment. Preventive measures should consist of frequent cleaning of cages, separating animals, avoiding grouping, raising floor with wire mesh so droppings can pass through, etc.

4. **Ear Mange.**—This is caused by a mite *Dermatocoptes* (*Psoroptes*) *cuniculi*. The disease is characterized by the formation of thick crusts or deposits on the inside of the ear.

The diagnosis is made by examining scrapings from the ear, unstained, under low power, for the mite.

Infected animals should be separated and treated locally. Remove the scabs and apply one of the following:

Mercuric chloride	1 part
Glycerin	100 parts
Ethyl alcohol (50 per cent)	200 parts

<div align="center">or</div>

Oil of caraway.......................	1 part
Almond oil	10 parts
Ethyl alcohol (90 per cent).............	3 parts

5. **Giardiasis.**—Giardia may be found quite commonly in the small intestines of rabbits. Whether or not they are harmful is questionable.

6. **Parasitic Cysts.**—Cysts of the dog tapeworm (*Taenia pisiformis*) are commonly found in the mesentery of the rabbit. They are spoken of as "bladder worms" and often occur in large numbers and occasionally in the liver.

The general health of the animal is not affected.

DISEASE OF MICE

Mouse Typhoid (Paratyphoid).—This is the commonest infection and is due to bacilli of the Salmonella group, especially *B. aertrycke* and *B. enteritidis.* Spontaneous epizootics occur, causing considerable loss; the mortality is high, ranging from 34 to 95 per cent.

Susceptibility varies in different breeds and strains of mice and is influenced by diet and environment.

The diagnosis is established by isolating an organism belonging to the Salmonella group from the spleen or other organs.

DISEASES OF RATS

1. **Lung Disease.**—This is a respiratory infection prevalent among albino rats. Young rats are not susceptible. The symptoms are loss of appetite, rhinitis, conjunctivitis and labored breathing; as the disease becomes chronic there is much depression. The mortality is high.

At autopsy the lungs are always affected; unresolved pneumonias, catarrhal bronchitis with bronchiectases, abscesses and pleurisy are common findings.

Various organisms of dissimilar groups have been isolated from these lesions, none of which have been recognized as the cause. The etiology therefore is still undetermined.

2. **Paratyphoid.**—This disease is due to members of the Salmonella group, particularly the *B. enteritidis* type. The symptoms in acute cases are diarrhea and bloody crusts around the eyes.

The lesions found at autopsy are swollen spleen, multiple necrotic foci, enlarged

lymph glands and Peyer's patches. **Chronic cases may fail to show any lesions.** The organisms can be readily isolated from the spleen and liver.

GENERAL DIRECTIONS FOR THE INJECTION OF ANIMALS

1. Select an appropriate size syringe that does not leak upon being tested with water. Nothing is more unsatisfactory than a leaking syringe, for not only may the hand become soiled, but an unknown quantity of inoculum is lost.

2. Remove the plunger from the barrel, and sterilize all the parts by boiling for at least several minutes. An all-glass syringe or a glass barrel and metal plunger are the most satisfactory. The old-fashioned syringe with washers and rubber-tipped plunger should find no place in a laboratory.
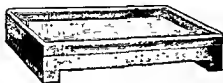
FIG. 25.—ANIMAL BOARD

3. After cooling, expel the water and load the syringe. This may be done by drawing the fluid directly into the syringe and measuring the dose by its markings or by pipeting the exact dose into a sterile Petri dish or capsule and drawing up in the syringe.

4. The animal should be fastened or held firmly and in an easy position. Everything should be in readiness, so that the injections may be given thoroughly and carefully. Various holders have been described; those shown in Figures 25 and 26 may be recommended.

5. In preparing the inoculum, care should be exercised that no solid particles enter the syringe. Aside from possibly blocking the needle and interfering with the injection, the subcutaneous injection of small fragments may do no particular harm, but in intravenous inoculation they may cause fatal embolism.

FIG. 26.—COCA-RAMSDELL BOARD

6. Air bubbles should be removed. The injection of small bubbles of air into subcutaneous tissues may cause no harm, but when injected into veins they may cause serious disturbances or immediate death. To avoid this, the syringe, after being filled, should be held vertically, with the needle uppermost. The needle should be wrapped in cotton soaked in alcohol and the piston of the syringe pressed upward until all the air is expelled from the barrel and the needle. If a drop of inoculum is forced out, it will be collected on the cotton, which should immediately be burned.

7. Injections should be slowly given.

8. When it is necessary to incise the skin in order to reach a vein, an anesthetic may be given. With superficial veins, and in subcutaneous inoculations, the injections may be given so readily and easily that no more pain can be felt than that which accompanies similar injections in human beings.

9. When it is necessary to remove the hair from the area to be injected, a small area may be clipped as closely as possible, followed, if necessary, by shaving. In guinea-pigs the hair may be plucked out; or clip the hair and apply a paste of equal parts of barium sulphide and cornstarch mixed with water. Leave this on for three or four minutes, wash thoroughly with warm water and dry with a towel. This is a particularly good method when large areas of skin are to be prepared. As it may cause irritation, it is well to remove the hair a day in advance of injection.

10. Before injection cleanse the skin with 70 per cent alcohol or wipe with 1:1000 mercurophen or other disinfectant.

TECHNIC OF INTRACUTANEOUS INJECTION

1. Select white animals or white areas if skin reactions are to be elicited as allergic reactions, diphtheria toxin reactions, etc.

2. Use a 1 c.c. syringe with No. 26 needle.

3. Prepare the skin. Pinch up a fold and insert the needle (lumen up) as superficially as possible. A raised, white, anemic spot showing the pits of hair follicles indicates a successful injection. Owing to thin skins of rabbits and guinea-pigs, the injection is by no means easy or simple and requires practice. The amount injected should not exceed 0.1 c.c.

TECHNIC OF SUBCUTANEOUS INJECTION

1. Injections are usually given in the median line of the abdominal wall or in the groin (Fig. 27).

2. Have the animal held firmly by an assistant or firmly secured to the animal operating table.

3. Clip the hair where injection is to be made—it is not always necessary to shave the area. Apply a 2 per cent iodine in alcohol solution.

4. Pinch up a fold of skin between the forefinger and the thumb of the left hand; hold the syringe in the right hand, insert the needle into the ridge of skin between the finger and thumb, and push it steadily onward until the needle has been inserted about an inch. Care must be exercised not to enter the peritoneal cavity. Relax the grasp of the left hand and slowly inject the fluid. If the skin is raised, this shows that the injection is subcutaneous. If it is not, the needle should be slightly withdrawn and inserted.

5. Withdraw the needle, and at the same time cover the puncture with a wad of cotton wet with alcohol. A touch of flexible collodion over the puncture completes the operation.

6. If a *solid* inoculum is to be injected, raise a small fold of skin with a pair of forceps, and make a tiny incision through the skin with a pair of sharp-pointed scissors.

7. With a probe, separate the skin from the underlying muscles to form a funnel-shaped pocket.

8. By means of fine-pointed forceps or a glass tube syringe, introduce the

inoculum into this pocket and deposit it as far as possible from the point of entrance of the instrument.

FIG. 27.—SUBCUTANEOUS INJECTION OF RABBIT
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

9. Close the wound with collodion and cotton. A single stitch with fine thread may be necessary.

TECHNIC OF INTRAMUSCULAR INJECTION

1. These injections are usually made into the posterior muscles of the thigh or into the lateral thoracic or abdominal muscles.

2. Clip away the hair over the selected area, cleanse, etc., as for subcutaneous injection.

3. Steady the skin over the selected muscles with the slightly separated left forefinger and thumb.

4. Thrust the needle of the syringe quickly into the muscular tissue and slowly inject the fluid.

TECHNIC OF INTRAVENOUS INJECTION

Rabbit.—1. The posterior auricular vein along the outer margin of the ear is better adapted than a median vein for this purpose.

2. If a number of injections are to be made, commence as near the tip of the ear as possible, as the vein may become occluded with thrombi, and subsequent inoculations may then be given nearer and nearer the root of the ear.

3. The animal should be held firmly, as the slightest movement may result in piercing the vein through and through and require reinsertion of the needle. This is accomplished satisfactorily by placing the rabbit upon the edge of the table and holding it firmly there by grasping the neck and front quarters, the assistant at the same time compressing the root of the ear with the thumb and forefinger.

4. If the hair is long, clip it.

5. The ear is struck gently with the fingers and washed with alcohol and xylol; the friction will render the vein more conspicuous.

FIG. 28.—INTRAVENOUS INJECTION OF RABBIT

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

6. The ear is grasped at its tip and stretched toward the operator, or the vein may be steadied by rolling the ear gently over the left index finger and holding it between the finger and thumb.

7. The inoculum should be free from solid particles and all the air excluded from the syringe. As a general rule, the amount injected should be as small as possible, and the temperature of the inoculum be near that of the body. If the syringe is filled shortly after sterilization, when it has cooled enough to be comfortably hot to the touch, the heat will warm the injection fluid and not be hot enough to cause coagulation.

8. Hold the syringe as one would hold a pen, and thrust the point of the needle through the skin and the wall of the vein until it enters the lumen of the vein (Fig. 28).

The wooden box shown in Figure 29 is very convenient for holding rabbits for intravenous injection or for bleeding from the ears.

9. Direct the assistant to release the pressure at the root of the ear, and *slowly* inject the inoculum. If the fluid is being forced into the subcutaneous tissue, which will be evident at once by the swelling which occurs, the injection must cease and another attempt be made.

10. The needle is quickly withdrawn, a small piece of cotton moistened with alcohol placed upon the puncture wound, and firm compression applied.

Wash the ear thoroughly with alcohol and water to remove xylol, otherwise a low-grade inflammation will follow, which will render subsequent injections more difficult.

Guinea-Pig.—1. The large superficial vein lying on the dorsal and inner aspect of the hind leg of the guinea-pig is well adapted for intravenous injection. Occa-
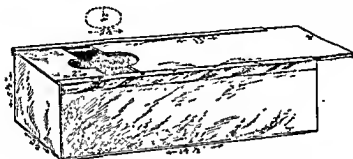
FIG. 29.—A WOODEN BOX FOR RABBITS

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

sionally, however, the vessel may run anteriorly. To use the above described vessel for intravenous administration, a special operating board is required. The board is similar to an ordinary animal board, except that the end to which the hind legs of the animal are tied has a U-shaped piece cut from it. The board is mounted near the center on an extension shaft which is fitted with two joints, the one at the end to which the board is attached being a ball-and-socket joint and the other an adjustable swivel joint. The shaft is screwed into a metal base which has sufficient weight to hold the board steady when placed in any position. A single operating board, however, may be used, as shown in Figure 30.

2. The procedure for making the injection is as follows: With the board properly placed in a horizontal position, the animal is tied to it securely, abdomen downward, by means of strings. The board is then placed in a vertical position and rotated on its vertical axis slightly so as to bring the dorsal aspect of the right hind leg into view. After clipping the hair from the leg and shaving it, the leg is lifted up slightly by the first or first and second fingers, and the vein dilated by suitable compression. The vessel can now usually be seen through the skin. A small incision, usually about one-fourth of an inch long, is made across the leg from the outer lower to the upper and inner aspect, but a trifle to the left of the vessel. The subcutaneous tissue is then pushed aside with fine-pointed forceps, thereby permitting the vessel to come into view.

3. The vessel is then entered directly or in the same manner as has been described for the rat—that is, by passing the needle of the tuberculin syringe through the fascia and muscles to the left of the vessel and then entering the vessel from the side. The vessel when dilated permits the ready entrance of a gauge No. 23 needle. However, the needle usually employed is a gauge No. 26, five-eighths of an inch in length. The needle is always introduced well into the lumen of the vein. If entrance into the vessel is direct, subsequent hemorrhage may be controlled readily by pinching it with a small forceps.

4. Injections may be also given in one of the external jugular veins as follows:
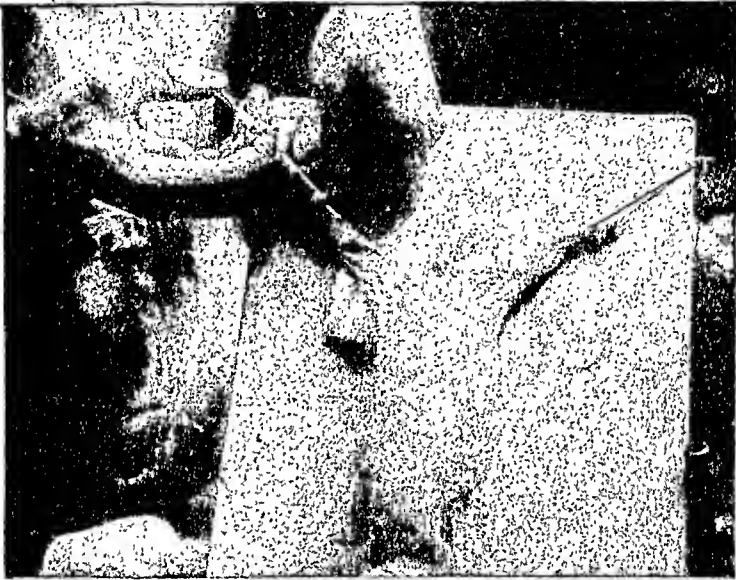
Fig. 30.—Roth's Method for Intravenous Injection of the Guinea-Pig
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

(*a*) A small roll is placed under the neck of the animal to render the operative area tenser and more easily accessible.

(*b*) A few drops of ether may be given by an assistant, although one soon learns to expose the vein quickly and there is practically no pain after the skin has been incised.

(*c*) Assistant is directed to hold the head backward in the median line.

(*d*) Pick up the skin just above and in the middle of the space between the shoulder and the tip of the upper end of the sternum—just above and about in the center of the area where a clavicle in the human would be situated. With small sharp scissors incise the skin for about one-half inch. Separate the subcutaneous tissue gently with forceps; a large vein at once comes into view. Gently dissect it free for about one-quarter inch.

(*e*) Pick up the vein with a pair of fine forceps, insert the needle of the syringe gently in the long axis of the vein, and slowly inject the fluid (Fig. 31).

(*f*) Withdraw the needle and apply firm pressure with a wad of clean gauze or

cotton. It is not necessary to tie off the vein. A stitch may be inserted to close the skin wound and flexible collodion applied.

White Mouse.—1. The lateral veins of the tail of the white mouse have been found best suited for intravenous administration purposes. The tail must be free from localized or generalized thickening of the epidermis so as to permit the ready entrance of a gauge No. 23 needle. The use of a rather long needle, 1 inch in length, is essential; it does not bend easily and therefore can be directed forward more readily than a smaller one.

Fig. 31.—Intravenous Injection of Guinea-Pig.

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

2. A mouse weighing between 15 and 20 grams practically always possesses a soft, pliable tail which can be used without any preparation. When a mouse weighing over 20 grams is used, the lateral veins of the tail are usually covered with rather dense tissue, which precludes their use unless the tail is immersed for about a half minute in warm water (about 50° C.). This procedure both softens the skin and dilates the underlying vessels so that the latter may be successfully used.

3. For holding the mouse, a small tin mailing tube attached to an iron stand

is employed (Fig. 32). One end of the metal mailing tube is fitted with a cork having at the circumference a V-shaped opening, which will admit the tail. The other end of the tube contains several small openings for the purpose of admitting air.

4. The mouse is grasped by the tail with the thumb and forefinger of the left hand (Fig. 33) and placed in the above-described metal mailing tube, and the cork is inserted so that the tail protrudes through the V-shaped opening. The tail is now
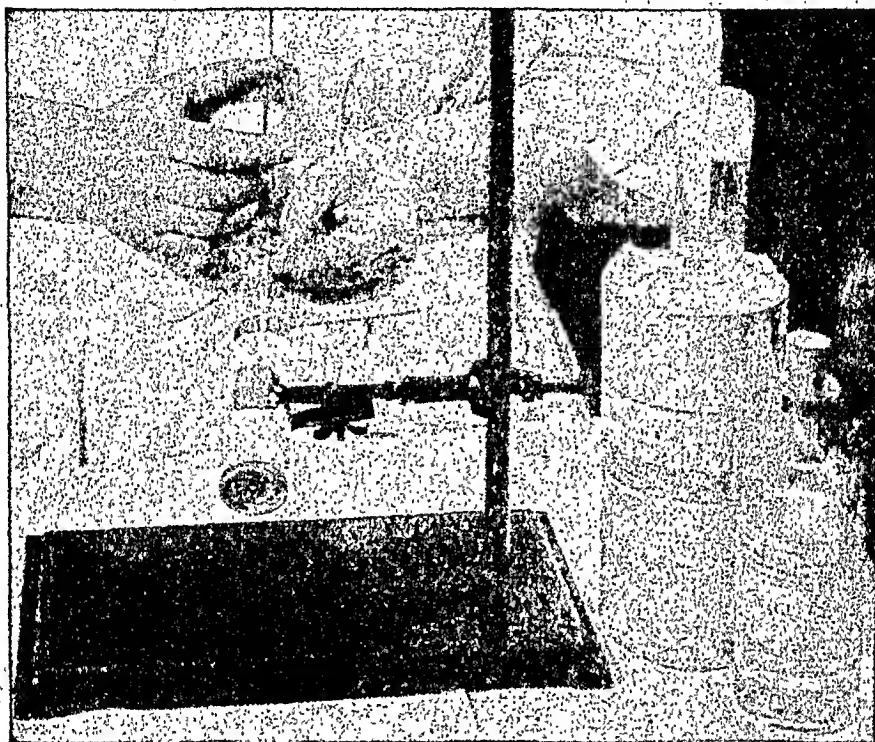
FIG. 32.—ROTH'S METHOD FOR INTRAVENOUS INJECTION OF THE MOUSE
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

straightened by gentle but firm traction and without twisting. The dorsal vein should then appear above, and each lateral to the left and right, respectively.

5. The syringe, usually a 1 c.c., all-glass, tuberculin type, graduated to 0.01 c.c., is balanced between the first and middle fingers of the right hand, the hand resting on the little finger; the thumb is thus free to operate the piston of the syringe. With the syringe held nearly parallel to the tail, the needle is pushed through the skin over one of the lateral veins (usually the left) and then anteriorly and downward into the vein. If an entrance into the vessel is not affected, either raising or lowering the point of the needle while advancing it further will usually succeed in locating the lumen of the vessel.

White Rat.—1. The animal is tied securely by the legs, back downward, to a flat operating board, by means of strings long enough to permit the hind legs to be lifted easily.

2. At the end of the board to which the head is tied are two glass pegs about 1 inch long set in at an angle in order to hold the string which is looped over the
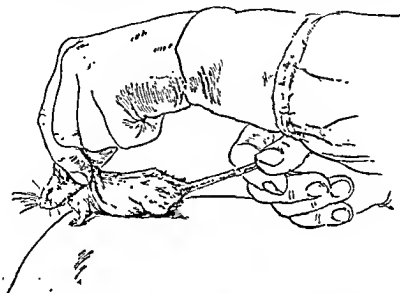
Fig. 33.—Manner of Grasping a Mouse
(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

front legs of the animal. Nails in the other end of the board receive the strings which are looped to the hind legs (Fig. 34).

Fig. 34.—Intravenous Injection of the White Rat Employing the Femoral Vein
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

3. After shaving the hair over the skin area covering the left saphenous vein, the left foot is grasped between the third and middle fingers of the left hand, and

an incision about one-fourth to one-half of an inch long is made about one-fourth of an inch to the left of and parallel to the vein. The skin is then rolled over to the right with the first finger of the left hand by drawing the skin on the back of the leg to the left. This will bring the vessel into view. An assistant then makes compression to dilate the vessel. If a syringe is used, it is preferable to employ a 1 c.c., all-glass tuberculin type, graduated to 0.01 c.c. and fitted with a gauge No. 26 needle, five-eighths of an inch in length. The syringe is balanced between the first and middle fingers of the right hand, the hand resting on the little finger; the thumb is thus free to operate the piston of the syringe. The needle is then

Fig. 35.—Intravenous Injection of the White Rat with Gravity Apparatus
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

passed through the fascia and upper surfaces of the muscles, about one-eighth of an inch to the left of the vein and almost parallel to it. Advancing the needle slightly farther, the direction is changed so that the needle will enter the vein from the side. After the injection is made, the skin which was pulled to the right to permit the vessel to come into view, is released and this skin flap and the muscles act as effective mechanical checks to hemorrhage, which is quite profuse, if the needle is inserted directly into the vein.

4. If a buret is employed and the injection made by gravity instead of by means of a syringe, a flexible rubber tube is attached to the buret, while the othe

end of the tube carries a glass tube which is drawn out and ground to fit a gauge No. 25 needle 1 inch long. The glass tube is handled in the same way as the syringe, and the vessel is entered in the same manner as described under the syringe method (Fig. 35).

Fig. 36.—Intravenous Injection of Rat Employing the External Jugular Vein
(From Kolmer. *Chemotherapy*, W. B. Saunders Co.)

5. An *external jugular vein* may be used instead and has the advantage of being larger.

The animal is tied to the operating board in the same manner and a wad of cotton placed under the shoulder. The assistant holds the head backwards and to one side with a piece of cotton, rendering the tissues of the operative area tense and firm (Fig. 36).

The skin is touched with alcohol and a small incision made just above and about in the center of the area where a clavicle in the human would be situated. The subcutaneous tissues are gently dissected and the vein exposed, which becomes very prominent with respiratory movements. It is well not to attempt entering the vein until it is thoroughly exposed, as otherwise one may infiltrate the tissues over and about the vein by failure to enter it properly. As soon as the injection has been given the animal is quickly released and the wound

Fig. 37.—Dog Operating Table

requires no attention as infection practically never occurs and healing is rapid.

Dog.—1. Dogs may be injected through the external jugular or popliteal veins. The animal should be fastened to the operating table (Fig. 37).

2. There is a small vein just beneath the skin in the median line, along the anterior surface of the leg, which is readily accessible. Clip away the hair and disinfect with iodine and alcohol. Direct the assistant to grasp the thigh just above the knee to distend the vein and prevent movement, and make a small incision directly in the median line. A small vein is seen at once. Dissect free or pick up gently with fine forceps and insert a small sharp needle. The injection can thus be readily given. Withdraw the needle, apply firm pressure, and insert a single stitch. Bind the wound with a few turns of a gauze bandage or seal with collodion and cotton.

TECHNIC OF INTRACARDIAL INJECTION

1. Guinea-pigs may be injected by the intracardial route instead of intravenously. The technic is not, as a rule, more difficult, and no ill effects are noticed. Not infrequently, however, attempts to inject in the heart fail, and frequent trials are not permissible on account of the danger of injuring the organ.

2. The animal is tied to the operating board, or held firmly by an assistant; an anesthetic may be given.

3. Determine the point of maximum pulsation to the left of the sternum by palpation, and quickly insert a thin, sharp needle at the selected area. A flow of blood indicates that the needle has entered the heart. Attach the previously filled syringe and slowly inject the contents.

4. Detach the syringe in order to make sure that the injection was intracardial as intended, which is indicated by a flow of blood; then quickly withdraw the needle. The puncture wound may be sealed with collodion.

TECHNIC OF INTRAPERITONEAL INJECTION

Rabbit.—1. Clip the hair and shave an area about 2 inches in diameter in the median abdominal line just below the umbilicus. Apply 2 per cent iodine in alcohol.

2. Direct an assistant to hold the animal firmly, head down. With the animal in this position the loops of intestine tend to sink toward the diaphragm, leaving an area above the bladder which is sometimes free of intestines (Fig. 38).

3. The syringe is grasped firmly and the needle inserted beneath the skin for a short distance in the direction of the head in the long axis of the animal, when the hand is raised and the needle forced forward through the peritoneum. When the peritoneum has been entered this is evidenced by a relaxation of the abdominal muscles. The needle is then withdrawn slightly and the injection made.

Guinea-Pig.—1. Direct an assistant to hold the animal firmly upon its back. This is better than fastening it to an operating table, for it permits relaxation of the abdominal wall when the injection is to be made.

2. Pluck the hair in the median abdominal line. A small area may be shaved, although this is not necessary. Disinfect with an application of iodine in alcohol.
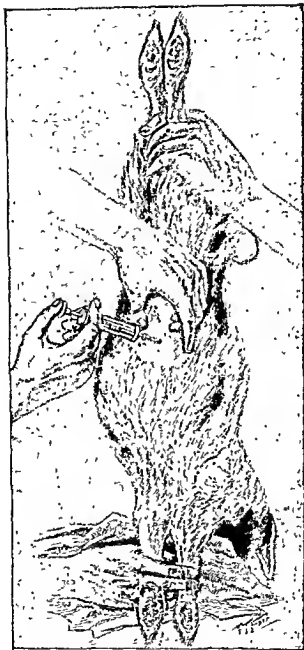
Fig. 38.—Intraperitoneal Injection of Rabbit

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

3. With the left forefinger and thumb pinch up the entire thickness of the abdominal parietes in a triangular fold, and slip the peritoneal surfaces over each other to ascertain that no coils of intestine are included.

4. Grasp the syringe in the right hand and insert the needle into the fold near its base.

5. Release the fold and inject the fluid. If a swelling forms, this shows that the
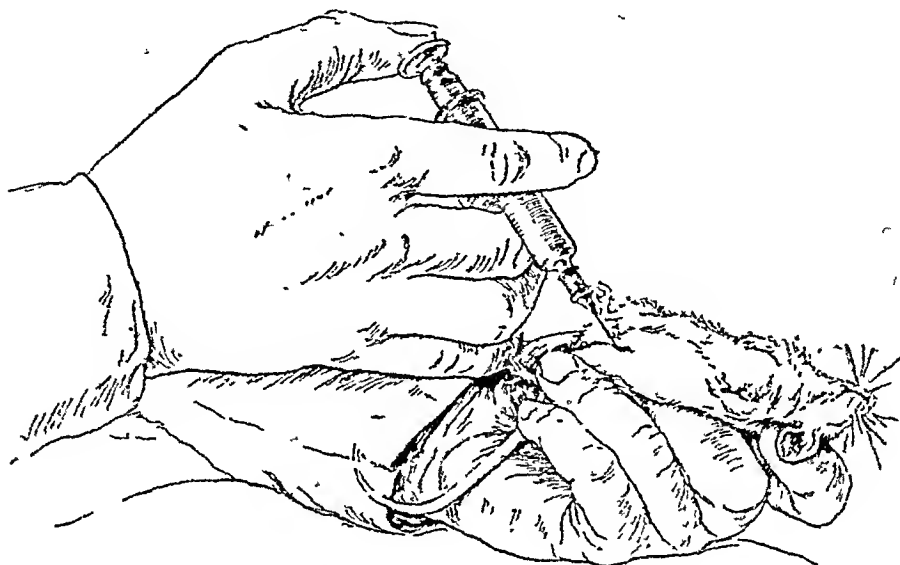
FIG. 39.—INTRAPERITONEAL INJECTION OF MOUSE
(From Wadsworth. *Standard Methods,* Waverly Press, Inc.. Baltimore.)

needle is in the subcutaneous tissues, and another attempt should be made to enter the peritoneum.

6. It may be difficult to pinch up the parietes without including the intestine. In such case straighten out the animal and stretch the skin between the left forefinger and thumb. Insert the needle obliquely until it is beneath the skin. A slight thrust suffices to pierce the peritoneum, when the abdominal muscles will be felt to relax. Withdraw the needle slightly and inject the fluid.

7. Seal the wound with a touch of collodion.

Mouse.—The technic is practically the same (Fig. 39).

TECHNIC OF SUBDURAL INJECTION

Rabbit.—1. Use a No. 18 gage needle which has been cut off and sharpened to about three-sixteenths of an inch in length, and a 1 c.c. syringe. Sterilize by boiling and fill with inoculum.

2. Shave or clip the hair over the site of injection, which is located a few centimeters posterior to and on a line with the outer canthus of the eye. In this region a small horizontal groove can be detected by feeling with the finger nail. The bone at this point is thin.

3. Place the needle in the groove and force it through the bone into the cranial cavity.

4. Inject the material slowly.

TECHNIC OF TESTICULAR INJECTION

Rabbit.—1. Sponge the scrotum with tincture of iodine.

2. Fill sterile syringe with material to be inoculated. Use a No. 22 gage needle about five-eighths of an inch in length.

3. Insert the needle through the skin of the scrotum into the substance of the testicle.

4. Inject the material.

METHODS FOR OBTAINING BLOOD FROM ANIMALS

Rabbit.—*From the Ear Veins.*—1. Flip an ear vigorously with the hand, and rub with xylol and alcohol. The xylol produces marked congestion and afterwards

Fig. 40—Method of Bleeding a Rabbit from the Ear

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

should be carefully removed with alcohol and water, as it produces a low-grade inflammatory reaction.

2. Puncture a marginal vein with a large needle. The blood will flow quickly in drops and practically any amount up to 10 c.c. or even more may be collected in a centrifuge or test tube (Fig. 40). For making preliminary tests of serum

iring immunization, 2 c.c. of blood are usually sufficient. Bleeding may be
iecked by making firm pressure over the puncture.

Another good method is to place the animal in the box shown in Figure 41,
irning it up on end so that the animal's head is down. This permits one to bleed
ie animal without assistance.

From the Heart.—1. Tie the animal securely to a board and clip the hair from
n area of the chest about one and one-half inches in diameter.

2. Determine the point of maximum pulsation and disinfect with tincture of
odine.

3. If 5 to 20 c.c. of blood are required, use a sterile syringe fitted with a No. 17
o 19 gage needle. If more than
20 c.c. are required or the animal
is to be "bled out," use a 200 c.c.
bottle with a rubber stopper fitted
with two pieces of glass tubing,
one connected with rubber tubing
for suction and the other con-
nected by means of heavy-walled
rubber tubing with the needle.
Sterilize in an autoclave.

4. Etherize lightly unless the
worker is experienced, when ether
may be omitted as it may cause
more discomfort than the punc-
ture.

5. Enter the needle at the point
of maximum pulsation, applying
gentle suction.

6. Approximately 20 c.c. of
blood can be taken from a 2000
gram rabbit at intervals of two or
three weeks.

From the Neck.—1. Clip the
hair from the neck and disinfect
with 70 per cent alcohol or 1 per
cent lysol solution; dry with a
towel.

Fig. 41.—Method of Bleeding a Rabbit from
the Ear

(From Wadsworth, *Standard Methods*, Waverly
Press, Inc., Baltimore.)

2. While an assistant holds the animal head down, rapidly sever the neck on
both sides with a razor or sharp scissors (avoiding the trachea and esophagus),
and collect the blood by a funnel into centrifuge tubes or a dish. Or the following
may be used: By means of a sterile knife the skin is cut longitudinally and the
neck muscles exposed for a considerable distance.

3. The animal is then held upright by the assistant over a sterile dish or a large
sterile funnel, emptying into a cylinder or 50 c.c. centrifuge tube.

4. The operator stretches the neck by carrying the head backward, and severs the large vessels on one or both sides of the neck with a sharp sterile scalpel or razor, avoiding opening the trachea and esophagus.

5. After bleeding, the dish is covered or the tube plugged and set aside for the serum to separate.

Guinea-Pig.—The animal is anesthetized with ether or stunned with a sharp blow and the large vessels of the neck on one side are exposed by a longitudinal incision. These are severed, and the blood is collected in a Petri dish or in a centrifuge tube by means of a funnel. Or by means of a sharp-pointed scissors

Fig. 42.—Method of Securing Blood from the Heart of a Guinea Pig
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

the vessels on one or both sides of the neck may be incised transversely at one cut, inserting the blade deeply and close to, but avoiding, the trachea and esophagus.

Blood may be obtained by aspiration from the heart of the living animal. A syringe fitted with a No. 20 or 22 needle is employed. The animal is fastened to a board or held by an assistant and lightly anesthetized. The point of maximum pulsation is determined and the needle slowly entered into the right chambers of the heart.

As a general rule, 5 to 15 c.c. of blood may be obtained by gentle suction, the amount depending upon the size of the animal. Large male animals (700 grams or more) are recommended and may be used every two to three weeks. After withdrawal of the needle the animal rapidly recovers, although occasionally bleeding may follow into the pericardial sac (Fig. 42).

Sheep.—Blood may easily be obtained from a freshly killed animal. The first flow of blood is discarded, and a portion of the remainder is collected in a large, sterile, thick-walled flask containing glass beads. By shaking vigorously the blood is defibrinated if one desires to obtain corpuscles, or the blood may be collected in a cylinder and defibrinated by whipping with glass rods.

It is usual, however, in large laboratories to keep a sheep and to remove the

FIG. 43.—METHOD OF BLEEDING A SHEEP FROM THE EXTERNAL JUGULAR VEIN, A. The needle here shown is reduced to a little more than half the actual size. (From Kolmer. *Infection, Immunity and Biologic Therapy.* W. B. Saunders Co.)

blood as it may be required. Small amounts may be obtained from the ear vein, larger quantities being secured from an external jugular vein in the following manner (Fig. 43):

1. One may do the bleeding alone, although the aid of an assistant is usually necessary. This is especially so if the animal is large, powerful and inclined to be vicious.

2. The sheep is thrown on its back, and the head is held on the knees of an assistant seated on a low box or stool.

3. The operator may straddle the animal to hold down the fore feet, although this is not necessary unless the animal is vicious.

4. The wool on the left side of the neck is clipped closely with scissors and alcohol applied.

5. The operator then grasps the neck low down with the left hand, and by means of the thumb exerts pressure over the base of the neck. The external jugular vein will be found in a groove between the omohyoid and sternomastoid muscles. Firm pressure over the base of the neck usually distends the vein, which may be seen or easily felt. After locating the vein the pressure should be released for an instant, when the distention will disappear. In this way the operator may be more certain that he has located the vein.

6. A sterile stout needle, at least 2 inches in length and provided with a trocar and special shank for firm grasping, is passed quickly into the distended vein in an upward and inward direction. It is essential that the needle be sharp, otherwise it will be turned aside by the wall of the vein. The end of the needle must not have too long a bevel, or the point will pierce the opposite wall before the body of the needle is well within the vein. The trocar is now removed, and blood collected in a flask or bottle and defibrinated with glass beads or rods. A short piece of rubber tubing may be attached to the needle. A suction apparatus is not needed because the flow of blood is good so long as pressure is preserved over the vein at the base of the neck.

7. When the required amount of blood has been secured, pressure is released and the needle quickly withdrawn. Bleeding ceases at once, and the neck is then washed with alcohol.

8. By this method the same vein may be used over and over again for several years. We have never known infection to occur, although the gradual formation of scar tissue about the site of puncture may interfere with the operation.

Horse and Cow.—1. Clip the hair on the side of the neck over the jugular vein.

2. Apply alcohol.

3. Apply pressure with thumb until the vein becomes prominent.

4. Insert a No. 16 to 18 gage needle first through the skin and then into the vein.

5. After collecting blood, remove needle and wipe with alcohol.

Hog.—1. Cleanse the tip of the tail and wipe with alcohol.

2. Lay the end of the tail on a block of wood and chop off about one-quarter to one-half inch with razor.

3. Allow the blood to run into a sterile container.

4. When required amount is obtained, tie a string around the tip of the tail to prevent further bleeding.

Dog.—1. Muzzle and lay on side.

2. Clip hair and then shave the side of the neck over the jugular vein.

3. Have the head extended and apply pressure over jugular vein until it becomes prominent. If the tissues around the vein are loose it is well to draw the tissues down at the same time the pressure is applied, thus rendering the vein immovable.

4. Insert the needle through the skin and then into the vein.

Fowl.—*From the Comb.*—Clip off a small piece of one of the points of the comb. Sufficient bleeding will occur to furnish blood for blood counts, hemoglobin estimations, smears, etc.

From the Humeral Vein.—1. Hold the bird on its side so that the breast is presented toward the operator. Turn the top wing back.

2. Insert the needle (No. 21 gage) into the humeral vein which is situated in the loose fascia on the inner side of the wing in the humeral region.

3. Withdraw the blood and place in tube as quickly as possible as the blood of fowls coagulates rapidly.

TECHNIC OF POSTMORTEM EXAMINATIONS

1. Autopsies should be performed as soon after death as possible, especially if bacteriological examinations are required.

2. Select suitable instruments and have a separate set for each step if bacteriological examinations are to be made (in which case boil all instruments for at least five minutes).

3. Fasten the animal on a board or tray exposing the ventral surface. Disinfect the hair with 1 per cent cresol and examine the skin at the point of inoculation for ulcerations, etc.

4. Incise the skin from the neck to the pubis. Cut the skin at right angles at the ends of the cut.

5. Separate the skin and lay the flaps back on each side, exposing the entire abdomen and thorax. Note the condition of the subcutaneous tissue, axillary and inguinal lymph glands.

6. Make cultures of the peritoneal fluid at this time by puncturing the peritoneal wall with point of capillary pipet or needle attached to a syringe.

7. With fresh instruments cut through the peritoneal wall from diaphragm to the pubis. Make right angle cuts to form flaps which can be laid back to expose the organs.

8. Examine and make necessary cultures of the abdominal organs.

9. With blunt-pointed scissors cut through the costal cartilages, making a V-shaped incision. Lay back the flap and expose the thoracic organs.

10. If a blood culture is to be made, lift the heart and hold it in position with a hemostatic forceps. Cut the pericardium and sear an area of the right ventricle with a hot instrument; make a short incision with a sterile scalpel, withdraw blood with a sterile pipet or a sterile loop to a suitable culture medium and make smears on slides.

11. If histological examinations are desired, remove small pieces of tissue and place them in a Zenker's solution or 4 per cent formalin; use five to six times as much solution as tissue.

12. Dispose of the animal by burning or place in a 1 per cent solution of crude cresol for disinfection.

13. Those who are not familiar with the anatomy of the various experimental animals should consult texts on this subject such as the following:

Bensley, A. B.   Practical Anatomy of the Rabbit, 3rd ed., P. Blakiston's Son
     & Co., Philadelphia, 1921.
Hunt, H. R.   A Laboratory Manual of Anatomy of the Rat, New York, 1921.
Schauder, W.   Anatomie der Impfsäugetiere, in Martin's Lehrbuch dere
     Anatomie der Haustiere, ed. 2, Stuttgart, 1923, 4:303.
Jaffe, R.   Anatomie u. Pathologie der Spontaner-krankungen der Kleinen
     Laboratoriumstiere, Berlin, 1931, p. 832.

CHAPTER III

METHODS FOR THE PREVENTION AND EMERGENCY TREATMENT OF LABORATORY ACCIDENTS

The modern laboratory worker is surrounded by many dangers, especially from infection in the conduct of necropsies and the handling of infectious material in bacteriological examinations, as well as from breaking glassware, scalds, burns, the accidental swallowing of corrosive poisons, the inhalation of poisonous fumes, etc. This is especially true of inexperienced workers who are likely to be ignorant of potential dangers or engage in conversation and other distractions while handling and measuring infectious material, cultures, acids, alkalis, etc.

PREVENTION OF ACCIDENTS

1. Good rubber gloves always should be worn in the conduct of necropsies of animals or individuals and the handling of fresh infectious tissues (anthrax, syphilis, tuberculosis, etc.); and great care should be taken against accidental pricking of the fingers with sharp edges of bone and needles as well as against cuts with knives, saws, etc.

2. Pipets employed for transferring or measuring cultures of virulent bacteria like tubercle, diphtheria, and typhoid bacilli, *B. abortus*, etc., should have the mouth ends plugged with cotton or a piece of rubber tubing with a glass mouth piece should be attached for filling and expelling. Various syringes are also available. Likewise in pipeting acids, alkalis and other poisonous solutions, the worker should use these precautions and exercise great care. It is particularly important to keep one's mind on the work and not to engage in conversation or other distractions.

3. The hands should be kept free of cuts and abrasions, particularly around the finger nails, and carefully washed with soap and water followed by immersion in a disinfecting solution after handling infectious material and before meals.

4. Table tops should be carefully wiped with a disinfectant solution after working with infectious material and it is sometimes advisable to work on a towel wrung out in a disinfectant like 2 per cent lysol or tricresol solution.

5. Pipets, test tubes, instruments, etc., employed in the examination of infectious material should be placed immediately in a disinfectant solution like 2 per cent lysol or tricresol or immediately sterilized by boiling.

6. In grinding dried bacteria a mask should be worn or the procedure conducted in a special hood insuring against the inhalation of dust.

7. Containers contaminated with sputum, feces, etc., or slides soiled with excreta

sive amounts of vaginal or urethral pus, etc., should not be handled at all but consigned to a disinfecting process.

8. Chemical work involving the production of irritating and dangerous fumes should be always conducted under hoods with good exhausts and ventilation.

9. Electric heaters and other electric apparatus should be frequently inspected and immediately repaired as a safeguard against short circuiting and fires. Bunsen burners should never be used around inflammable material. Ether, alcohol and the like should be carefully kept away from all possible contact.

10. All laboratory workers should be immunized against diphtheria if Schick-positive; also against typhoid and paratyphoid fevers. Cowpox vaccination is advisable every few years and after every contact with smallpox. Good general health should be maintained at all costs as an effective means for keeping natural immunity and resistance at a high level.

EMERGENCY TREATMENT OF ACCIDENTS

Cuts and Needle Pricks.—1. Remove all foreign matter, such as pieces of glass, dirt, etc.; then apply 3.5 per cent tincture of iodine, taking care that the tincture reaches all crevices of the wound.

2. If the cut is slight or does not bleed copiously, bandage, placing a small pad of gauze directly over the wound and bandaging tightly enough to stop the flow of blood. If a small cut does not stop bleeding from the pressure bandage alone, apply peroxide of hydrogen and bandage with a pad of gauze over the wound.

3. If the cut is severe and bleeds copiously, apply a tourniquet to check the bleeding. If the cut is in an artery, indicated by the blood being a bright scarlet and flowing in an intermittent stream, the tourniquet is to be placed between the cut and the heart. If in a vein, shown by dark, purplish blood, the tourniquet should be placed between the capillaries and the wound. Under no conditions should a tourniquet be allowed to remain in place for more than two hours at a time.

4. Needle pricks should be squeezed to promote bleeding, carefully washed with hot water and soap and treated with tincture of iodine or 1:500 metaphen or mercurophen solutions.

Burns.—*From Flames or Hot Objects.*—1. Dress with butesin picrate ointment, and, if serious or covering a large area, bandage over the dressing. Dressing should be completely changed at least once a day.

2. Blisters forming from burns should be opened and drained by puncturing in at least two places near the edge and pressing out the liquid. The puncture may be made with a flame-sterilized needle or razor blade. When changing dressing, any blisters present should be again drained.

From Chemical Agents.—1. Burns from *strong acids*, bromine, chlorine, phosphorus or other material of acid character, are washed first with large quantities of water, then with 5 per cent sodium bicarbonate or 5 per cent ammonium hydroxide solution, dressed and bandaged as above.

2. Burns from *strong alkalis*, sodium hydroxide, metallic sodium or potassium

or other materials of alkaline nature, are washed first with large quantities of water, then with 5 per cent boric or acetic acid solution, dressed or bandaged as above.

3. Burns from *phenol* are washed immediately with strong alcohol, then dressed and bandaged, if necessary.

IN THE EYE.—Flush first with large amounts of water. Then, if due to *acid* material or formaldehyde, flush with 5 per cent sodium bicarbonate solution. If due to *alkaline* material, flush with 5 per cent boric acid solution. In either case a drop or two of castor, cotton seed, or olive oil is allowed to remain in the eye as a soothing agent.

Scalds.—Blisters that have formed are to be drained as above; then the injury is dressed and bandaged, using suitable neutralizing agents if due to acid or alkaline materials, as prescribed for burns.

Swallowing of Mineral Acids.—1. Wash out the mouth immediately with large amounts of water and alkaline solutions like decinormal sodium hydroxide, milk of magnesia, etc.

2. Give calcined magnesia, white magnesia, milk of magnesia, or lime-water *immediately*, mixed in milk or any mucilaginous fluid that will act as a demulcent. Repeat the dose at short intervals until it may be inferred the poison is neutralized. Do not give carbonates as antidotes for mineral acids. Oleaginous and mucilaginous fluids should be given freely, even as vehicles for the antidotes. In the case of strong sulphuric acid, water, if given at all, should be given in large quantities, on account of the heat developed. Ice may be given to relieve thirst and pain. Stomach tube and emetics should not be used. Or give a teaspoonful of *universal antidote* in a small glass of warm water. This antidote is made by mixing two parts of pulverized charcoal, one part of magnesium oxide and one part of tannic acid. It is well to keep a supply on hand.

Swallowing of Caustic Alkalis.—1. Wash out the mouth immediately with large amounts of water and weak acid solutions.

2. Administer a weak acid, such as 5 per cent acetic acid, vinegar, or lemon juice, until it may be inferred the alkali has been neutralized; or give a teaspoonful of universal antidote (see above) in a small glass of warm water. Give butter, olive or cotton seed oil, or other oils or fats to form soaps.

3. Assist vomiting by drafts of tepid water.

Swallowing of Phenol and Phenolic Compounds.—1. Wash out the mouth immediately with dilute alcohol (30 to 40 per cent).

2. Give *immediately* 4 ounces of alcohol mixed with 4 ounces of water, or one half pint of whisky, and remove from stomach by use of emetic, preferably of mustard (tablespoonful in enough water to make a thin cream). If stomach tube is used, it must be with great care.

Inhalation of Corrosive Gases.—1. Remove patient to fresh air, and place prone, face down, with head slightly lower than the chest, so that vapors may drain from the lungs.

2. Permit inhalation of vapors of acetic acid if ammonia is active agent, and of dilute ammonia if acid vapors are being treated.

3. Inhalation of vapors of alcohol or ether soothes the respiratory tract.

4. *Toxic headaches* due to the inhalation of vapors of various materials may be combated by removal to fresh air, administration of 5 to 10 grains of aspirin, and allowing the patient to rest for a time.

5. For *hydrogen sulphide* fumes inhale ammonia from 5 per cent ammonium hydroxide, or inhale fresh air containing a small proportion of chlorine. Administer milk, white of egg in water, olive or cotton seed oil, etc.

Swallowing of Virulent Cultures.—1. Accidental contaminations of the mouth with cultures of staphylococci, streptococci, pneumococci, etc., are not dangerous although it is advisable to immediately rinse well with hot water and disinfectant like 1:5000 metaphen or mercurophen, 1:2000 bichloride of mercury, diluted hydrogen peroxide, etc.

2. Virulent diphtheria cultures are much more dangerous. The above measures should be immediately employed. If Schick-negative, there is little or no danger of infection. If Schick-positive, infection may follow unless a prophylactic injection of 1000 units of antitoxin is taken.

3. The swallowing of typhoid, cholera and dysentery cultures also carries some danger, especially in the case of freshly isolated cultures. The mouth should be immediately disinfected as described above. In the case of typhoid cultures, it may be advisable to undergo vaccination unless this had been done within two years.

4. Great care is also required in working with *B. abortus* (*Micrococcus melitensis*). In case of contamination immediate immunization with vaccine is recommended following the cleansing measures recommended above. Great care is also required when working with *Bact. tularense.*

Contamination with Syphilitic Material.—1. In case of needle pricks or cuts while removing chancre or other syphilitic material for dark-field or other examinations, squeeze to promote bleeding; then wash carefully with *hot* water and *plenty of soap.* Dry and thoroughly apply 33 per cent ointment of calomel or metallic mercury or 1:1000 solution of bichloride of mercury (ointments preferred). Renew the applications twice a day for at least three days.

2. Contamination of the hands with the blood of syphilitic patients while taking blood for the Wassermann test, giving injections, etc., is rarely dangerous and not at all unless there are needle pricks, cuts or abrasions. We have never seen or heard of accidental infection in this way. In case of contamination of cuts and pricks with blood, it is advisable, however, to use the measures described above.

3. We have never heard of or seen syphilitic infection following accidental contamination of the mouth with syphilitic serum or cerebrospinal fluid while conducting the Wassermann and other tests. The manipulations involved in the preparation of serum and especially heating at 55° C. for ten minutes or longer, are almost sure to kill the spirochetes, even if they were present, as *Spirochaeta pallida* is very susceptible to heat.

Spinal fluids, however, especially from paretics, are potentially more dangerous,

particularly if contamination occurs during spinal puncture or soon thereafter. If needle pricks or cuts occur, the measures given above should be applied. If spinal fluid is accidentally taken into the mouth during total cell counts or other examinations, it will suffice to rinse thoroughly with water and 1:500 metaphen or mercurophen or 1:1000 bichloride of mercury.

SECTION II

CLINICAL PATHOLOGICAL METHODS

CHAPTER IV

METHODS FOR THE EXAMINATION OF THE BLOOD

APPARATUS FOR BLOOD EXAMINATIONS

Principles.—Next to urine examinations it is probable that red and white corpuscle counts with hemoglobin estimations and differential leukocyte counts are the most frequently conducted laboratory examinations in clinical pathology. Yet a comparison of the counts on the same patient at the same time by different workers is apt to show widely different results due to inaccuracies in technic and
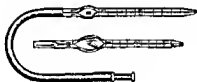
FIG. 44.—THOMA DILUTING PIPETS   FIG. 45.—TRENNER AUTOMATIC PIPETS
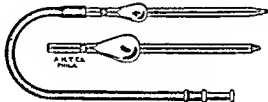
apparatus. Cheap diluting pipets, counting chambers and cover glasses are responsible for a great deal of error and only the better grades are recommended, preferably with the United States Bureau of Standards certification. Pipets with broken tips are especially likely to be inaccurate. It is recommended that only the best apparatus be employed with scrupulous attention to technical details.

1. Thoma, (Fig. 44) or Trenner (Fig. 45) diluting pipets are recommended for red and white corpuscle counting, the latter particularly for inexperienced workers if difficulty is experienced in stopping the flow of blood exactly at the 0.5 mark on the capillary stem.

2. All pipets should be guaranteed to be within the tolerance for accuracy established by the United States Bureau of Standards of ± 3 per cent for the principal interval. Pipets with the Bureau of Standards certification are recommended, although probably too expensive for routine work. It is advisable, however, for every laboratory to have at least one certified red corpuscle and leukocyte pipet with which new pipets may be compared before the latter are accepted for work.

3. While many different counting chambers are in use, the Levy with the improved Neubauer ruling (Fig. 46) is recommended; also the Levy-Hausser with

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bakelite holder and double Neubauer ruling. At slight extra cost these may be purchased with the United States Bureau of Standards certification and every laboratory should have at least one for comparative counts with new chambers before the latter are adopted for routine work.

4. Accurate blood cell counting requires a cover glass with optically plane surfaces. *The curvature of an ordinary cover glass as used in bacteriological work is such as to render an otherwise*
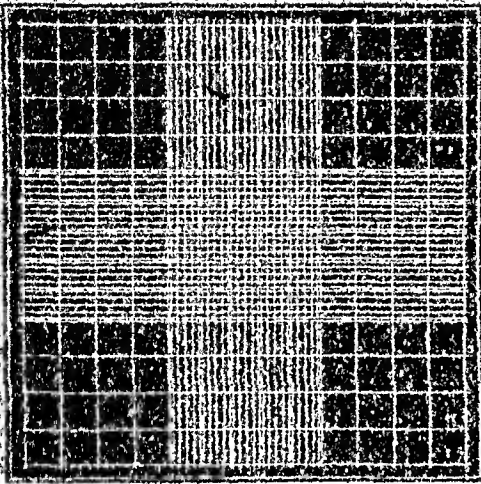
FIG. 46.—ENTIRE AREA OF IMPROVED NEUBAUER RULING

FIG. 47.—MARBEL BLOOD CELL CALCULATOR

perfect counting chamber absolutely useless from the standpoint of accuracy. For very little extra cost, cover glasses may be obtained with the United States Bureau of Standards certification, and these are recommended for the best work. The Hausser reinforced precision glasses are recommended for the counting chamber method of standardizing vaccines.

5. The Marbel blood cell calculator (Fig. 47) is highly recommended for red and white corpuscle counting and for differential leukocyte counting. The Veeder hand counter, while not as elaborate, is less expensive and very useful (Fig. 48).
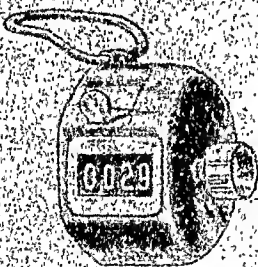
METHODS FOR OBTAINING BLOOD

FIG. 48.—VEEDER HAND COUNTER

1. Blood is usually obtained by pricking a finger or the lobe of an ear; the finger is to be preferred.

2. All apparatus (pipets, diluting solutions, slides and hemoglobinometer) should be in readiness.

3. The simple (Fig. 49) and the spring (Fig. 50) lancets are quite serviceable though a straight Hagedorn needle No. 6 ground to a short angle and stuck in a cork as shown in Fig. 51, is quite satisfactory. The "sticker" should be broad rather than narrow, so that an ordinary needle or pin is unsatisfactory.

4. The finger should be warm with a free circulation of blood (immersion in warm water may be required). If cold and clammy, too much squeezing is required with resulting error.

5. Wash off the side of the last phalanx or the end of the middle finger with alcohol and *thoroughly dry. Do not prick a wet finger.*

6. Squeeze to steady the part and lessen the pain sense while making a quick prick (Fig. 51).

Fig. 49.—Simple Blood Lancet

7. If the tip of the finger is used, lance across rather than parallel with the lines of the skin.

8. **Do not squeeze too hard near the prick** as this will dilute the blood with tissue "juice" and lead to error. The prick should be deep enough to give a free flow with gentle squeezing one-fourth to one-half inch from the site.

Fig. 50.—Spring Blood Lancet

9. Wipe away the first drop or two of blood.

10. It is advisable to take blood first for the hemoglobin estimation followed by the red and white corpuscle pipets and the preparation of smears for differential leukocyte counts.

11. Well up a good-sized drop of blood for each preparation and especially for

Fig. 51.—Pricking Side of Finger with Hagedorn Needle

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

the leukocyte pipet, which requires much more than the red corpuscle pipet. See Figure 52.

12. Be sure to fill each pipet accurately with the proper diluting fluid immediately after taking blood (Fig. 53).

13. Immediately after filling a pipet with diluent, shake it for thorough mixing (Fig. 54).

14. If the pipets are to be carried any considerable distance to the laboratory,
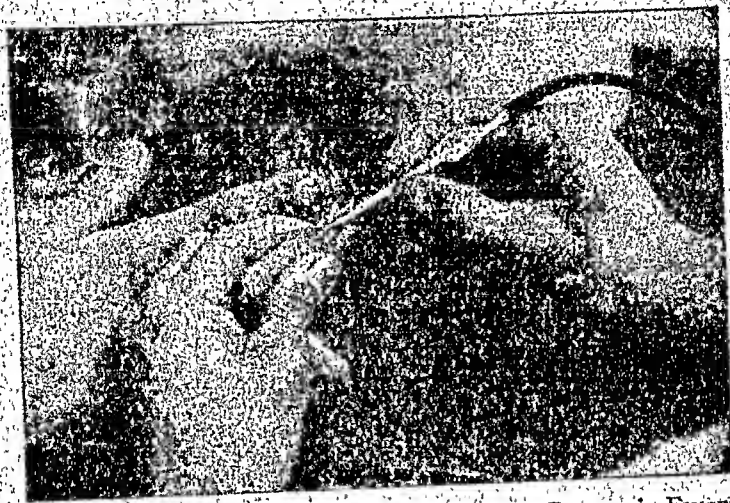
FIG. 52.—TAKING UP BLOOD INTO PIPET FROM PATIENT'S FINGER

Note that the operator's right hand is steadied by touching a finger against the finger of the patient. (From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)
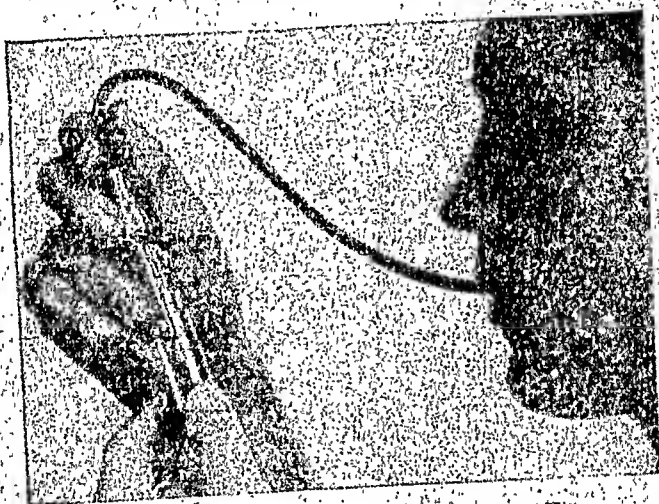
FIG. 53.—FILLING PIPET WITH DILUTING FLUID

The pipet is held high enough so that the marks are level with and are plainly visible to the eye. (From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)
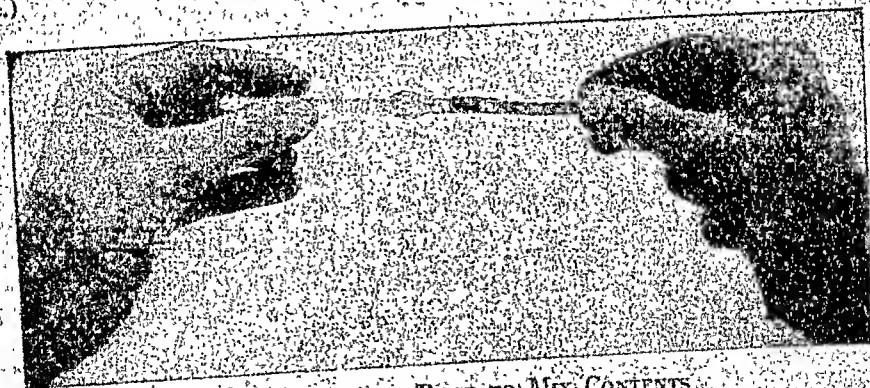
FIG. 54.—REVOLVING PIPET TO MIX CONTENTS

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

stretch a rubber band over the ends (Fig. 55) or one of the special devices (Figs. 56 and 57) to prevent the contents from escaping.

15. Prepare two or more smears on *perfectly clean grease-free and polished slides.*[1] Touch the end of a slide to a large drop of blood as shown in Figure 58; then spread the drop with a second slide as shown in Figure 59. As soon as the

Fig. 55.—PIPET SEALED WITH A BROAD RUBBER BAND

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore.)

Fig. 56.—THE TRENKER CLOSURE

blood has spread entirely across the end of the spreader slide (held at an angle of about 45 degrees) with a rather quick movement, push (do not pull) the spreader toward the other end of the under slide (Fig. 60). If made too slowly the spread will be too thin. Allow the blood to dry.

16. Blood smears may also be made on coverglasses (Fig. 61). They should be perfectly clean and free of grease. Take up a small drop of blood on one without touching the surface of the skin and place it on the second in such manner that the corners do not overlap. As soon as the blood spreads out between the glasses draw them apart in a plane parallel to their surface. Dry in the air. This method is preferred by many clinical pathologists.

17. A combination of the side and coverglass methods is that of Beacom in which smears are prepared as follows: a small drop of blood is placed near the end of a clean slide and a coverglass is dropped on; if the blood does not flow
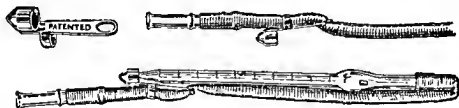
Fig. 57.—THE DRUMMOND PIPET HOLDER

out properly, very gentle pressure may be used. The slide is then held on a table with the left hand, and the first two fingers of the right are placed near the edges close to the left end of the coverglass. Then with an even, fairly rapid pull it is slid along the slide in the direction of its long axis with only enough pressure to keep the fingers from slipping from the spreader. This leaves a smooth, even smear on the slide for examination.

[1] Place slides in glacial acetic acid for fifteen minutes; wash in distilled water; place in 95 per cent alcohol for five minutes; dry with clean towel; pass through Bunsen flame before using.
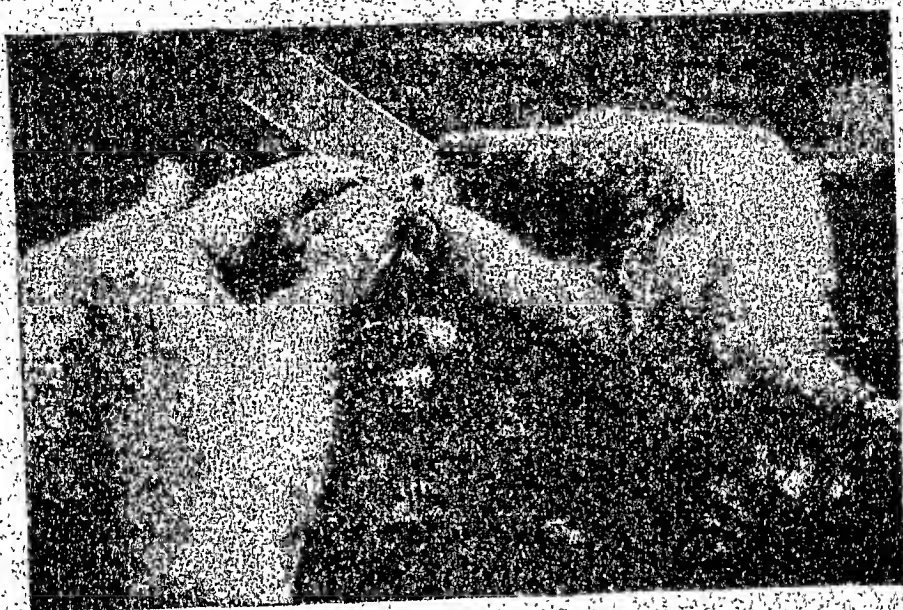
FIG. 58.—TAKING BLOOD FOR A SMEAR

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore.)
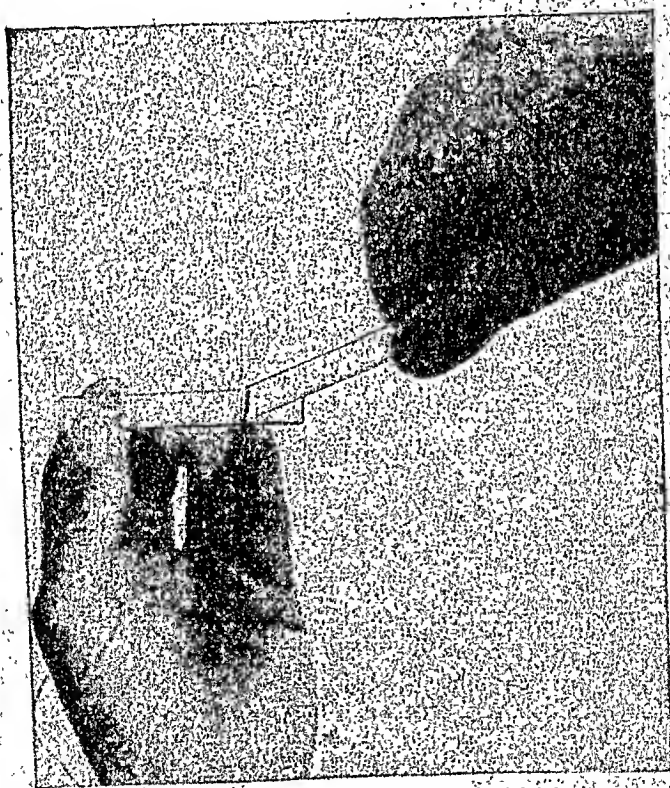
FIG. 59.—SLIDES HELD IN PROPER POSITION FOR SPREADING BLOOD

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore.)
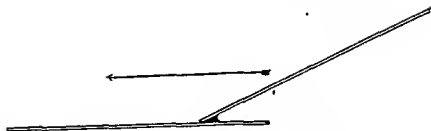
FIG. 60.—PROPER ANGLE FOR HOLDING SLIDES

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

METHOD OF USING OXALATED BLOOD FOR HEMATOLOGICAL EXAMINATIONS

According to Osgood, Haskins and others, oxalated blood may be used for the following examinations if the latter are made within the designated hours after collection:

	Hours
Hemoglobin estimation	24
Red cell count	24
Platelet count	3
Red cell volume	24
Color index	24
Volume index	3
Saturation index	3
Icterus index	4
Van den Bergh test	4
White cell count	24
Making the smear for different count	1
Peroxidase test	3
Fragility test	3
Sedimentation rate	1

1. Prepare a month's supply of test tubes containing 2 milligrams of dry *potassium oxalate per c.c. of blood to be taken*, by measuring into each, with a buret, 0.1 c.c. of 2 per cent potassium oxalate per c.c. of blood to be taken and evaporating to dryness in a dry sterilizer. Keep corked. (For small laboratories 1 drop of saturated potassium *oxalate is sufficient for 5 c.c. of blood and has a negligible dilution effect.*)

2. Draw blood from vein by usual technic, but tourniquet should be released if more than two minutes are required for securing sufficient blood.

3. *Remove needle from syringe before running blood into oxalate as hemolysis will result if blood is forced through the needle.*

4. Stopper test tube (never use cotton) and shake at once by holding test tube horizontally in left hand and tapping other end with right hand.
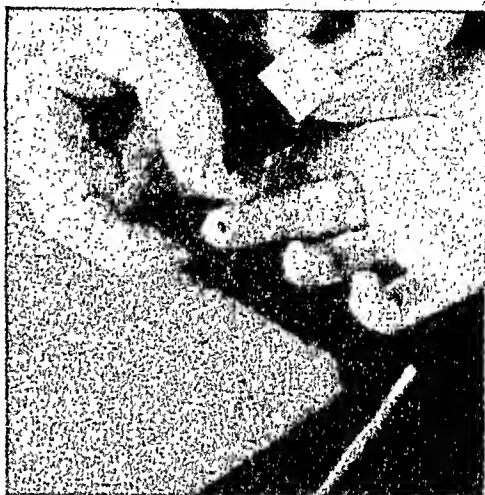
FIG. 61.—THE PREPARATION OF BLOOD FILMS BY THE COVER GLASS METHOD (HADEN)

A, the cover glass (⅞ inch square, No. 2) is grasped at the adjacent corners with the thumb and forefinger of each hand; B, the drop of blood is touched with the cover glass held in the right hand; C, the cover glass carrying the drop of blood is quickly placed parallel on the cover glass held in the left hand; D, the cover glasses are then drawn apart with a sliding motion, care being taken to keep them parallel. The films are allowed to dry in the air and are then ready for staining. The drop of blood must be globoid on the finger tip and just large enough to cover the cover glass when properly spread.

5. The blood must be thoroughly mixed in this manner *immediately* before samples are withdrawn for any test.

6. Samples should be taken directly from the test tube—not from blood poured out on a slide or watch glass.

7. The tube must be kept stoppered at all times when not in use.

8. The time limits given above should be observed if the most accurate results are desired, although as a rule a slightly longer time will not introduce clinical error.

The advantages of this method are that if during the study of the blood further hematological work is deemed desirable, it may be done on the same sample. Repeated estimations are possible for checking results. If an unusual or interesting blood picture is encountered, as many slides as are desirable can be made for future reference or for teaching purposes without again disturbing the patient.

NORMAL VALUES AND RANGES

With the desire to aid those who use blood tests clinically, many of the average or mean normals have been purposely omitted and a range of normality substituted. The figures selected for the ranges are representative of the researches of recent years. In some instances the ranges have been expanded slightly at both ends so that round numbers could be used. *The span is great enough to include about 95% of normal individuals and yet narrow enough to avoid inclusion of any which might be considered definitely abnormal.*

TABLE I
NORMAL RANGES FOR HEMATOLOGY

	Men	Women	Children (6–13 yrs.)
Erythrocyte count (millions per c.c.)	4.5 to 6.0	4.0 to 5.5	4.2 to 5.8
Hemoglobin (gm. per 100 c.c.)	13 to 20	11 to 18	10 to 11
Packed cells (c.c. per 100 c.c. of blood)	36 to 52	35 to 46	31 to 41
Volume index*	0.80 to 1.00	0.80 to 1.00	0.62 to 0.82
Mean corpuscular volume (cubic microns)	80 to 100	80 to 100	62 to 82
Color index*	0.80 to 1.00	0.80 to 1.00	0.61 to 0.82
Mean corp. hemoglobin (micromicrograms)	27 to 32	27 to 32	20.4 to 27.6
Saturation index*	0.9 to 1.2	0.9 to 1.2	0.9 to 1.2
Mean corp. hemoglobin conc. (gm. per 100 c.c. cells)	33 to 38	33 to 38	
Diameter of erythrocytes (microns)	5 to 10	5 to 10	
Sedimentation rate (mm. in 1 hr.)†...	0 to 8	0 to 10	
Reticulocytes (per cent.)...	0.1 to 1.5	0.1 to 1.5	
Platelets (thousands per c. mm.)	200 to 500	200 to 500	
Leukocytes (thousands per c. mm.)	5 to 10	5 to 10	

*The ranges given for the volume color and saturation indexes are applicable only when the figures recommended as convenient arbitrary normals are used

†The range given applies to nearly all methods although some have reported lower figures

‡The ranges for children are calculated from figures obtained by Osgood and Baker. *Am. J. Dis. Child.* 1931 **50** 313.

Since the ranges vary according to age, sex, location, etc., and methods employed it is recommended that each laboratory whenever possible establish its own ranges At the same time the mean values should be determined for use in calculating the volume and color index.

Wherever possible the actual amount or number is given in the place of a percentage of a certain number or standard.

CLASSIFICATION of ANEMIA or NUMBER VOLUME AND HEMOGLOBIN CONTENT OF ERYTHROCYTES

	NORMAL	TYPES OF ANEMIA							
MEAN CORPUSCULAR VOLUME IN PER CENT OF NORMAL (RELATIVE CORPUSCULAR HEMOGLOBIN INDICATED BY INTENSITY OF SHADING)									
INDICES									
NUMBER INDEX	0.90-1.10	<0.080	<0.080	<0.080	<0.090	<0.090	0.90-1.10	<0.090	>1.10
VOLUME INDEX	0.90-1.10	>1.10	>1.10	>1.10	0.90-1.10	0.90-1.10	0.90-1.10	<0.090	<0.090
COLOR INDEX	0.90-1.10	>1.10	0.90-1.10	<0.090	<0.090	<0.090	<0.090	<0.090	<0.090
SATURATION INDEX	0.90-1.10	0.90-1.10	<0.090	<0.090	0.90-1.10	0.90-1.10	0.55	0.71	0.71
TYPICAL EXAMPLE									
CELL COUNT	5 MILLIONS	2.0	3.0	2.50	3.50	3.20	3.00	3.50	5.60
VOLUME INDEX	1.00	1.50	1.25	1.20	1.00	0.95	0.95	0.70	0.70
COLOR INDEX	1.00	1.40	1.00	0.84	0.95	0.70	0.60	0.50	0.50
SATURATION INDEX	1.00	0.95	0.80	0.70	0.95	0.75	0.63	0.71	0.71
CLASSIFICATION OF BLOOD	NORMOCYTIC AND NORMAL BLOOD	HYPERCHROMIC MACROCYTIC AND MACROCYTIC ANEMIA	HYPERCHROMIC MACROCYTIC AND MACROCYTIC ANEMIA	HYPOCHROMIC MICROCYTIC AND MICROCYTIC ANEMIA	HYPOCHROMIC NORMOCYTIC AND NORMOCYTIC ANEMIA	HYPOCHROMIC NORMOCYTIC AND NORMOCYTIC ANEMIA	HYPOCHROMIC MICROCYTIC AND MICROCYTIC ANEMIA	HYPOCHROMIC MICROCYTIC AND MICROCYTIC ANEMIA	HYPERCHROMIC AND ANEMIA

FIG. 62.—THE CLASSIFICATION OF THE ANEMIAS ON NUMBER, VOLUME AND HEMOGLOBIN CONTENT OF RED BLOOD CELLS

(Courtesy of Dr. Russell L. Haden.)

TABLE II

Normal Ranges of Blood Cellular Elements in Domestic and Laboratory Animals[1]

Species	Erythrocytes (millions)	Hemoglobin (Gm. per 100 ml.)	Erythrocyte Volume (%)	Platelets (thousands)	Leukocytes (thousands)	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Horse	5.5–8.7	10.5–16.4	30–44	35–500*	5–11	3,000–6,900	50–600	0–100	1,200–5,000	100–1,150
Cattle	5.0–10.0	8.0–15.0	30–50		5–12	1,200–4,800	10–1,800	0–100	2,700–6,900	150–1,800
Sheep	8.0–14.7	9.0–15.3	40–50		1–10	700–6,000	500–700	0–200	2,000–9,000	50–800
Swine	5.0–9.0	9.0–16.0		100*	1–20	2,100–10,000	50–2,000	0–200	3,200–12,000	50–2,000
Goat	9.0–19.0	12.7–15.2			5–14	2,100–3,350	0–1,100	0–600	2,100–11,250	50–600
Dog	5.5–8.5	12.5–17.2	10–30	250–450	5–15	3,600–13,000	100–2,000	0–100	600–6,000	50–600
Cat	7.0–10.0	9.0–17.8			5–10	3,000–21,700	100–3,000	0–120	1,200–13,200	100–2,100
Monkey	5.0–7.0	12.5–13.0			7–25	2,100–12,500	50–1,250	0–100	3,200–13,750	50–2,700
Rabbit	4.5–7.0	10.1–15.6		125–424	7–12	1,200–4,500	0–200	30–500	1,200–6,300	30–3,000
Guinea-Pig	4.5–6.0	13.6–17.3		200–1,000	5–23	1,800–10,000	100–3,000	0–100	2,100–11,000	50–2,000
Rat	7.0–10.2	15.6–19.0		200–900	6–16	1,200–6,000	0–600	0–100	1,000–12,000	20–1,000
Mouse	8.0–11.0	13.6–17.3		500–1,200	3–12	600–10,000	0–550	0–100	2,200–15,000	130–350
Chicken	1.5–4.5	8.0–11.0		45–53*	16–40	1,000–16,000	100–1,000	200–1,600	8,000–21,000	50–1,650
Frog*	0.4–0.6	6–16.5			10–33	200–2,600	2,600–10,000	700–2,600	6,000–22,000	1,000–6,000

* Too few counts reported for accurate determinations of ranges.

1 Compiled by A. H. Craige, Jr., V.M.D., School of Veterinary Medicine, University of Pennsylvania.

For convenience as a ready reference the normals for most of the commonly used blood tests are given on page 60 and a chart (Fig. 62) for aid in the classification of the anemias.

As various blood examinations are sometimes required in the course of medical research employing laboratory animals and since veterinarians are employing blood counts with increasing frequency in the diagnosis of disease of the lower animal, a table of normals for domestic and laboratory animals is given on page 62.

FIG. 63.—MULTI-PLE ASPIRAT-ING NOZZLE

METHODS FOR CLEANING BLOOD APPARATUS

1. Pipets and counting chambers should be cleaned immediately or soon after using.

2. Draw water through pipets until all traces of blood and serum are removed.

3. Without drying, draw through alcohol or acetone.

4. Draw through ether (may be omitted if acetone is used).

5. Draw air through until the pipet is dry (if properly dried the bead in the bulb should be freely movable).

6. If blood has dried in the stem of a pipet, remove it with horsehair or fine stiff wire and fill pipet with antiformin, nitric acid or bichromate-sulphuric acid cleaning fluid and allow to stand overnight: then clean thoroughly as described.

7. The multiple aspirating nozzle for attachment to a pump (Fig. 63) or the Haden cleansing apparatus (Fig. 64) are very convenient and time-saving for the simultaneous cleaning of one to four pipets.

8. The ruled area, the surface of the slide and the cover glass of the counting chamber should be cleaned immediately after use with water and dried with a soft lint-free cloth. If this is not done the lines will become partly obliterated with débris. If diluted blood has been allowed to remain on the slide or the ruling becomes indistinct, it may be necessary to immerse the slide in decinormal sodium hydroxide or in one of the solutions mentioned above for cleaning pipets. The Levy-Hausser chamber can also be washed with alcohol and ether without damage.
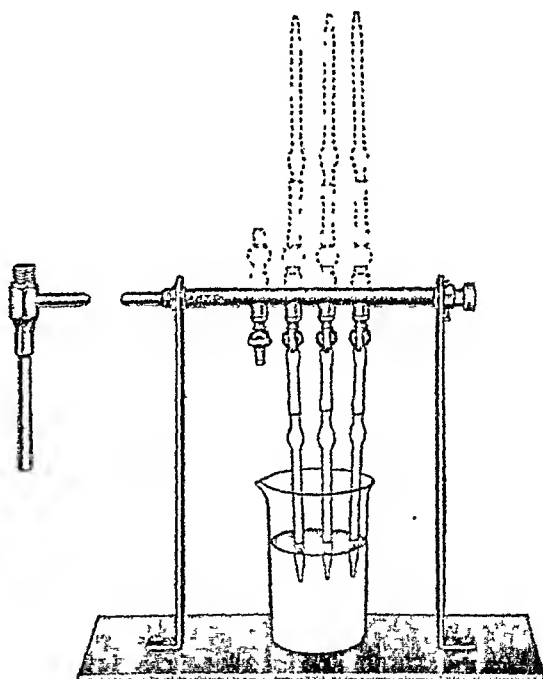
FIG. 64.—HADEN CLEANSING APPARATUS

METHODS FOR THE ESTIMATION OF HEMOGLOBIN

Principles.—1. None of the methods in common use can be recommended from the standpoint of absolute accuracy. Owing to a variety of methods and standards the estimation of hemoglobin in per cent is often of the least value in the routine blood examination. Widely varying results may be reported therefore on the same individual examined in different laboratories. *But much of this inaccuracy and misunderstanding could be overcome by expressing the results as grams of hemoglobin per 100 c.c. of blood.*

2. It is not possible as yet to recommend any simple method of routine work as a standard.

3. The most accurate methods available at present are that of Van Slyke for determining the oxygen capacity of the blood and that of Wong for determining the iron content. These, however, are not sufficiently simple for routine work but are valuable for checking the accuracy of clinical methods.

4. *It is recommended that reports show hemoglobin in grams per 100 c.c. of blood instead of in per cent.* If 16.6 grams per 100 c.c. is acceptable as equivalent to 100 per cent, to convert grams into per cent multiply the reading by 6, which gives a satisfactory percentage for clinical purposes.

5. Since the normal hemoglobin differs with age, sex, altitude and time of day, any range of normality will be somewhat flexible. The normal ranges given in the following table are recommended for clinical purposes:

TABLE III

NORMAL RANGES OF HEMOGLOBIN IN GRAMS ACCORDING TO AGE AND SEX

	Male		Female	
Age	Minimum	Maximum	Minimum	Maximum
1–13 days	17	28	18	28
0.5–2 months	11	26	13	22
3–5 months ..	10	17	12	16
6–11 months ..	8	16	11	16
1–3 years	9	14	8	14
4–15 years	10	14	10	14
over 15 years	13	20	11	18

Sahli's Method.—1. Fill the graduated tube of Sahli's hemometer with decinormal solution of hydrochloric acid to the mark 10 (Fig. 65).

2. *After cleansing finger tip or lobe of ear with alcohol, make a puncture with lancet and gently compress* (*the blood should flow freely*); wipe off first drop, then fill pipet to the mark 20 with blood.

3. Immediately place the blood in the graduated tube containing the hydrochloric acid solution. Remove the last trace of blood by drawing the solution up in the pipet and expelling several times.

4. Dilute the mixture in the graduated tube with water until it has the same

tint and intensity of color as the standard tube. This is best done by adding a few drops at a time and comparing after each addition.

5. When both tubes are of the same color, note the figures on the tube at the *bottom of the meniscus.*

6. The newer tubes show both per cent and grams of hemoglobin per 100 c.c. of blood.

7. Accurate results demand that the inside diameters of the graduated tube and the tube containing the standard solution shall be the same and that the pipet and graduated tube be accurately marked. These specifications can be met without unreasonable extra expense. Of even more importance is the correct color of the standard solution. The tinted tubes now available are usually accurate to within 5 to 10 per cent and may be used either by day or night. The Hellige hemometer (Fig. 66) is highly recommended for use in this method.

8. A new standard color tube should be checked by the Van Slyke or Wong methods before being used. If there is more than 10 per cent error it should be discarded. Otherwise

FIG. 65.—SAHLI HEMOMETER

the error can be adjusted by adding or subtracting from the readings obtained.

Haden's Method.—*This employs the new Haden-Hausser hemoglobinometer* (Fig. 67) in which hemoglobin is also converted into acid hematin and the color compared with a permanent color scale of tinted glass.

1. Draw blood to the mark 0.5 of a leukocyte pipet and decinormal hydrochloric acid to 11 (gives a 1:20 dilution). If the hemoglobin is below 50, draw blood to 1 and the acid to 11 (gives a 1:10 dilution).

2. Shake well and allow to stand for thirty minutes.

3. Blow out several drops and allow the blood to run into the channel of the slide at the end of the cover glass. The channel fills by capillarity. A thin uniform film will also extend by capillarity from the dilution channel and cover the comparator slide above the color standard. This insures a light transmitting surface common to both dilution channel and standard.

4. A color-matching reading is now made according to directions accompanying the instrument and the results expressed in grams of hemoglobin per 100 c.c. of blood.

Osgood-Haskins Method.—1. Place exactly 1 c.c. of well-mixed oxalated venous blood in a 100 c.c. volumetric flask.

2. Add 40 c.c. of distilled water and allow to lake.

3. Add 50 c.c. of a fifth normal solution of hydrochloric acid; mix well and make up to 100 c.c.

4. Heat a portion of this mixture in a test tube at 55° to 60° C. for seven minutes or longer.
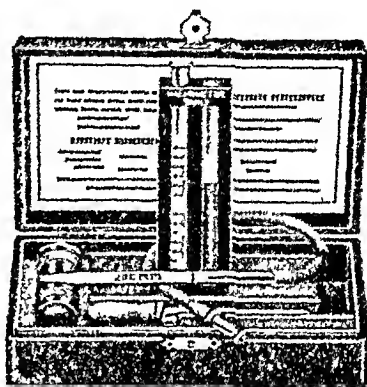
5. Place some of the standard solution in a colorimeter cup and set it at 15 mm., and compare the unknown with the standard:

STANDARD SOLUTION

Ferric sulphate .... 32 gm.
Chromic sulphate .... 80 mg.
Water (distilled) to make 100 c.c.

This standard should be checked with a previously prepared correct standard. This can be purchased from either Hynson, Westcott and Dunning, Baltimore, Maryland, or the Shaw Supply Co., Portland, Oregon.

6. Make three or four readings and take the average. Take the temperature
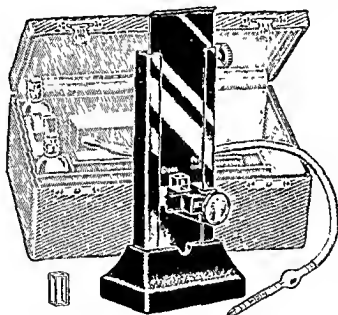
FIG. 66.—HELLIGE-WINTROBE HAEVOMETER

of the standard. Reference must now be made to a table which is furnished with the standard from which the percentage of hemoglobin is read directly for various colorimetric readings, and for different temperatures of the standard. A reading of 100 per cent represents 13.8 gms. per 100 c.c. when the standard is set at 15 mm. with a temperature of 15.5° C. and the unknown is read at 10.5 mm.

Osgood recommends that the first normal blood that is examined each week be kept as an acid hematin standard in a brown bottle, with the temperature

of the standard as originally read marked on the bottle. This solution can then be used as the standard for all subsequent determinations which are made during that week.

Photo-electric-cell Method (Sanford, Sheard and Osterberg).[2]—1. Dilute 0.1 c.c of blood in 20 c.c. of 0.1 per cent solution of sodium carbonate, thus making a 1:200 solution of oxyhemoglobin. This is most conveniently and most
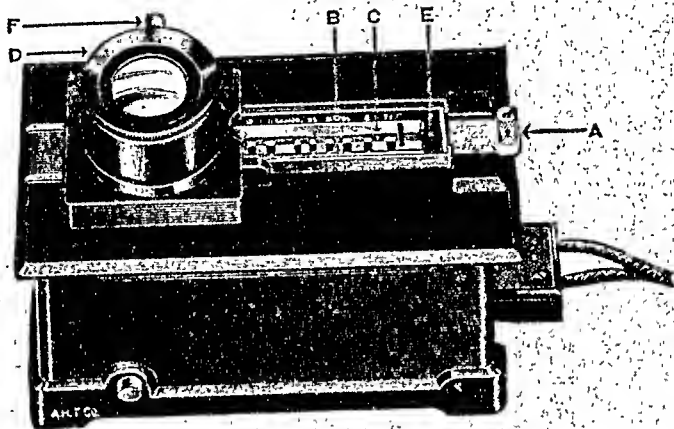
Fig. 67.—Haden-Hausser Hemoglobinometer.

A, Movable carrier; *B*, Comparator slide; *C*, Cover glass; *D*, Reading microscope; *E*, Wedge-shaped channel; *F*, Shutter.

accurately done, by making the dilutions from a sample of blood immediately after it is obtained by venipuncture.

2. The photelometer has a green glass filter in front of the photronic type of photo-electric cell. This filter transmits light in its maximal intensity at that portion of the spectrum where the maximal absorption occurs in one of the oxyhemoglobin bands. The light intensity through a standard spectroscopic cell, which is filled with 0.1 per cent sodium carbonate, is first adjusted with an iris diaphragm so that the reading on the meter is 100.

3. The specimen of diluted blood is placed in a similar spectroscopic cell, is then moved into the path of light in the carrier, and the reading is made on the meter (Fig. 68).

4. This lower reading really represents the decrease in current from the photronic cell which is the result of the light absorption of oxyhemoglobin in the green portion of the spectrum. This *reading is translated directly* into a value for grams of hemoglobin per 100 c.c. of blood by referring to a chart which is prepared individually for each instrument by the manufacturer, based on oxygen-capacity determinations.

This photelometer has proved very satisfactory, especially in laboratories in which a large number of routine determinations are made. Its only disadvantage is in the cost which is somewhat more than a colorimeter. However

[2] A. H. Sanford, C. Sheard, and A. E. Osterberg, *Am. J. Clin. Path.*, 1933, 3:405.

the instrument may be used as a colorimeter or a nephelometer; in fact there are many uses for it other than as a hemoglobinometer. The method is rapid and accurate. There is no attempt to match colors; therefore, subjective errors are avoided.

METHOD FOR COUNTING ERYTHROCYTES

Principles.—Blood is diluted exactly 1:200 with a special pipet, using an isotonic diluting fluid for the preservation of the corpuscles. The diluted blood
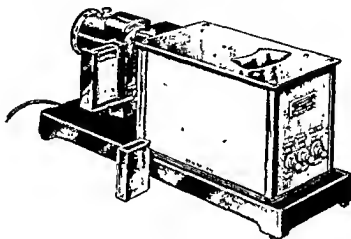
FIG. 68.—CENCO-SHEARD-SANFORD PHOTELOMETER

is then placed in a special counting chamber and the cells in 0.00025 c.mm. are counted. This figure is then multiplied by 4000 and then by 200 (the dilution factor) to obtain the number of erythrocytes in 1 c.mm. of undiluted blood. This procedure constitutes the method used to report the results of the erythrocyte count.

Leukocytes are likewise present and may be distinguished from erythrocytes, but as a general rule they are included in the count; the number present, however, is ordinarily so small that the inaccuracy is of little or no importance.

A pathological decrease is called oligocythemia or anemia; an increase is called polycythemia.

Procedure.—1. Draw blood up exactly to the 0.5 mark of the Thoma pipet marked 101. If the Trenner automatic pipet is used, draw blood by suction until the stem is nearly full and then discontinue suction and allow the blood to automatically reach the extremity.

2. Immediately draw up diluting fluid to the mark 101, thus making a dilution of 1:200 in either pipet, while rotating the pipet between the thumb and forefinger. Use 0.85 per cent saline solution or either of the following (Hayem's preferred):

HAYEM'S DILUTING FLUID

Water (distilled)	200.0 c.c.
Sodium chloride c. p.	1.0 gm.
Sodium sulphate (crystals)	5.0 gm.
Mercuric chloride	0.5 gm.

TOISSON'S DILUTING FLUID

Neutral glycerin	30.0 c.c.
Water (distilled)	160.0 c.c.
Sodium sulphate (crystals)	8.0 gm.
Sodium chloride c. p.	1.0 gm.
Methyl violet	0.025 gm.

3. The diluting fluid should be crystal clear and filtered if necessary to be free of artefacts.

4. The ruled area of the Levy-Hausser counting chamber and the cover glass must be carefully cleaned and absolutely free from dust or lint.

5. Place the cover glass in position over the ruled area, using gentle pressure to insure accurate adjustment. The Levy-Hausser chamber is provided with a pair of clips to prevent any movement during the count. While continuing pressure on the cover glass, slide the centrally placed clip into position simultaneously.

6. Close the tip by means of the thumb. Sharply kink the rubber tubing over the other end and place the second finger over the kinked tubing. Trenner pipets are more fragile than the Thoma pipets and when filling, cleaning or attaching rubber tubing, the capillary stem should be held between the thumb and forefinger to avoid strain on the bulb. Rotate the pipet well for several minutes, holding in a horizontal position, and finally shake sidewise.

7. Expel the fluid from the stem of the pipet and *without loss of time* touch a drop to the end of the polished surface bearing the ruling, allowing the drop to flow under the cover glass. The suspension should not flow into the moats on either side, nor should any bubbles form under the cover glass.

8. Allow about three minutes for the corpuscles to settle.

9. Examine with 4 or 16 millimeter objective. Center the light and reduce its volume by lowering the condenser and partially closing the diaphragm.

10. Locate the finding line which leads to the ruled-off area. *Carefully avoid touching the cover glass with the lens, as this would disturb the corpuscles and lead to error in the count.*

11. When correctly focused, the corpuscles are sharply defined and the rulings appear as well-defined *black* lines (Fig. 69).

When incorrectly focused, the ruled furrows appear as *white* lines, and the corpuscles, which lie above the plane of the ruled surface, are out of focus (Fig. 70).

12. The counting slide will be found to have a number of small squares marked upon it. The size of these small squares is 0.05 millimeter by 0.05 milli-

meter or 0.0025 sq. mm. When the cover slip is in place there is a chamber formed measuring 0.1 millimeter in depth. Therefore the small squares are in reality cubes measuring 0.00025 c.mm. (0.05 × 0.05 × 0.1 millimeter = 0.00025 c.mm.).

It is impracticable to count the number of cells in 4000 of these small cubes, which would give the number of cells per c.mm. of diluted blood, so that a smaller number of cubes should be chosen.

13. Count the number in 80 of the small squares. It will be noted that the small squares are separated into groups of 16. Five of these groups therefore
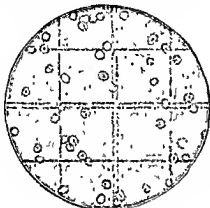
Fig. 69.—Correct Focusing   Fig. 70.—Incorrect Focusing
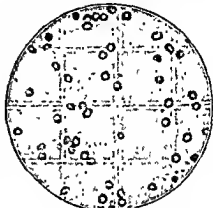
contain 80 small squares. Do not count those cells touching the lower and right-hand lines but include all touching the upper and left-hand lines.

14. Divide the number of cells found in 80 small squares by 80, thus determining the average number per small square or in 0.00025 c.mm.

15. Multiply the average number of cells per 0.00025 c.mm. by 4000 to determine the number of cells in 1 c.mm. of diluted blood, and by 200 to determine the number in 1 c.mm. of undiluted blood.

16. *Therefore in routine work count the number of cells in 80 small squares and add four ciphers.*

Sources of Error.—The following sources of error must be kept in mind and carefully avoided: (a) Inaccurate dilution due to faulty pipets or technic; (b) too slow manipulation, allowing a little of the blood to coagulate; (c) inaccuracy in the counting chamber and especially in its depth due to inaccurate cover glass, faulty manufacture, loosening of parts, etc.; (d) presence of yeasts and other artefacts in the diluting fluid; (e) delay in filling counting chamber after shaking pipets; (f) uneven distribution of the cells.

METHOD FOR DETERMINING THE VOLUME OF PACKED ERYTHROCYTES

Principles.—The estimation of the volume of packed cells in whole blood is chiefly used for the purpose of determining the volume index. It may also aid in

determining the degree of anemia. Changes in the size of erythrocytes, as well as in their number, will be detected.

The volume of packed cells in 100 c.c. of blood is normally as follows:

Men .... 36 to 52 c.c.
Women .... 35 to 46 c.c.

Finger Method.—1. Prick the finger as for blood count.

2. Gently compress until a large drop of blood collects.

3. Draw the blood up in a Van Allen hematocrit tube to the mark 10 (Fig. 71).

4. Draw the diluting fluid (1.4 per cent sodium oxalate) into the bulb of the pipet until it is about one-third full. Then mix by twirling between the fingers.

5. Place the tube in holder (Fig. 72) or wide rubber bands can be stretched over the ends.

6. Centrifuge the tubes at about 2500 revolutions per minute for about one-half hour. Remove the tubes and read the volume of cells according to the markings on the stem which is divided into 10 units of 10 divisions each. Each division represents 1 per cent.

7. Centrifuge again for five minutes and make another reading. If the two readings

FIG. 71—VAN ALLEN
HEMATOCRIT TUBE

FIG. 72.—SPRING
SEALING CLIP

are similar, it is taken as the per cent of red cells to whole blood. If, however, the reading is lower than the first the tube is again centrifuged for five minutes. This is continued until two readings are identical. With a little experience one soon learns the time and speed required.

Haden-Sanford Method.—1. Make venous puncture with a sterile needle and syringe and withdraw exactly 5 c.c. of blood.

2. Immediately transfer the blood to a tube carrying exactly 1 c.c. of 1.4 per cent solution of sodium oxalate. The Sanford-Magath tube is recommended (Fig. 73). The capacity is 6 c.c. so that the graduations in 0.1 c.c. are well separated and the level of the packed cells easily seen.

3. Mix well and centrifuge for sixty minutes at about 2500 revolutions per minute or until maximal packing of cells has taken place.

FIG. 73.—SAN-
FORD - MAGATH
HEMATOCRIT
TUBE

4. Or mix the blood thoroughly and place in a graduated centrifuge tube or in any straight tube in which the column of blood can be measured. The Sahli tube used in estimating hemoglobin is very handy for this purpose as the graduations can be used for reading the volume. Fill tube to the 100 per cent mark.

5. Centrifuge and read as described under the finger method making allowance for the dilution.

METHOD FOR DETERMINING THE VOLUME INDEX

Principle.—The volume index is the ratio of the mean corpuscular volume to an arbitrary normal.

In pernicious anemia in relapse a majority of the cells are larger than normal, hence the volume of packed cells will be greater than in the same unit of normal blood with the same cell count. The reverse is true in most cases of so-called secondary anemia. In acute losses of blood the cells are normal in size because of the fact that there has been no disturbance in their production for a day or two after the hemorrhage.

Procedure.—1. Determine the volume of packed cells per 100 c.c. of blood.

2. Make an erythrocyte count.

3. Calculate the volume index using the following formula:

$$\frac{\text{volume of packed cells per 100 c.c. of blood}}{\text{number of erythrocytes per cubic millimeter}} \div \frac{\text{mean normal packed cells per 100 c.c. of blood}}{\text{mean normal of erythrocytes per cubic millimeter}} = \text{Volume Index}$$

or

$$\frac{\text{per cent of normal packed cells}}{\text{per cent of normal erythrocyte count}} = \text{Volume Index}$$

Since there are no generally accepted figures for the mean normal packed cells and erythrocyte count, it becomes necessary either to adopt figures obtained by others or conduct a series of determinations on normal individuals and determine the mean. The figures obtained can be used for the above calculation. In the case of the erythrocytes some authorities have adopted five million as an arbitrary normal. The mean normals will vary according to sex, age, etc.

4. If for any group of normals, appropriately homogenous with respect to age, sex, race, etc., the mean normal is used which is consistent with one's own technique and experience, then the normal range of the volume index for that group will be 0.90 to 1.10. The value taken as normal for the mean cell volume should always be stated.[3]

5. If for simplicity of calculation and uniformity of practice one is content to accept as convenient arbitrary normal values 50 c.c. as normal for packed cells per 100 c.c. of blood and 5 million for the normal erythrocyte count, the following formula may be used:

$$\frac{\text{volume of packed cells per 100 c.c. of blood}}{\text{first two numbers of the erythrocyte count}} = \text{Volume Index}$$

If the erythrocyte count is under one million, the first number is used.

[3] For assistance with the mathematical calculations of the blood indices we are indebted to Dr. J. Harold Austin, Department of Research Medicine, University of Pennsylvania.

The normal range for volume index so calculated for adults is 0.80 to 1.00. For children between the ages of 4 and 13 years the normal range is 0.63 to 0.82.

When the volume index is calculated according to the latter method it is just another way of determining the mean corpuscular volume. The result is expressed in units of 100 cubic microns. It is therefore necessary to multiply the result by 100 to obtain the number of cubic microns per cell. This method is similar to the original method described by Capps in which he multiplies the numerator and denominator by 2 to express them in terms of percentage. The omission of this step in the calculation does not effect the results.

METHOD FOR DETERMINING THE MEAN CORPUSCULAR VOLUME

Principle.—The mean or average size of the individual erythrocyte in cubic microns may be calculated from the volume and the number of erythrocytes in a given quantity of blood. It has the same clinical significance as the volume index.

Procedure.—1. Determine the volume of packed cells per 1000 c.c. of blood (see page 71).

2. Make an erythrocyte count.

3. Divide the number of c.c. of packed cells per 1000 c.c of blood by the number of millions of erythrocytes per c.mm.:

$$\frac{\text{volume of packed cells per 1000 c.c. of blood}}{\text{erythrocytes in millions per cubic millimeter}} = \text{Mean Corpuscular Volume (cubic microns)}$$

4. The normal range for adults is from 80 to 100. The range for children between 4 and 13 years of age is from 62 to 82.

METHOD FOR DETERMINING THE COLOR INDEX

Principle.—The color index is the ratio of the mean corpuscular hemoglobin relative to an arbitrary normal.

This index usually closely parallels the volume index.

Procedure.—1. Determine the grams of hemoglobin per 100 c.c. of blood (see page 65).

2. Make an erythrocyte count.

3. Calculate the index by using the following formula:

$$\frac{\text{grams of hemoglobin per 100 c.c of blood}}{\text{number of erythrocytes per cubic millimeter}} \div \frac{\text{mean normal grams of hemoglobin per 100 c.c. of blood}}{\text{mean normal of erythrocytes per cubic millimeter}} = \text{Color Index}$$

or

$$\frac{\text{per cent of normal hemoglobin}}{\text{per cent of normal erythrocyte count}} = \text{Color Index}$$

Since there are no generally accepted figures for the mean normal hemoglobin and erythrocyte count, it becomes necessary either to adopt the figures obtained by others or conduct a series of tests on normal individuals and determine the mean. The figures obtained can then be used for the above calculation. In the case of the erythrocytes some authorities have accepted 5 million as the arbitrary normal. The mean normals will vary according to age, sex, etc.

4. If for any group of normal individuals appropriately homogenous with respect to sex, age, race, etc., the mean normal is used which is consistent with one's own technique and experience, then the normal range of the color index for that group will be 0.90 to 1.10. The value taken for normal for mean hemoglobin should always be stated.

5. If for simplicity of calculation and uniformity of practice one is content to accept as convenient arbitrary normal values 16.6 grams as the normal number of grams per 100 c.c. of blood and 5 million as the normal for the erythrocyte count, the following formula may be used when the count is 1 million or more:

$$\frac{3 \times \text{grams of hemoglobin per 100 c.c. of blood}}{\text{first two numbers of the erythrocyte count}} = \text{Color Index}$$

If the count is under 1 million erythrocytes, then the first number is used.

6. The normal range is from 0.80 to 1.00. The advantage of this method is its simplicity and the results are comparable with all others using this same method. Readings in grams can be converted into percent by multiplying the number of grams per 100 c.c. of blood by 6. When the color index is calculated according to this method it is just another way of determining the mean corpuscular hemoglobin. The result is expressed in units of 33.3 micromicrograms.

METHOD FOR DETERMINING THE MEAN CORPUSCULAR HEMOGLOBIN

Principle.—The mean or average amount of hemoglobin by weight per cell is calculated and expressed in micromicrograms (10^{-12} gm.). It has the same clinical value as the color index (see page 73).

Procedure.—1. Determine the hemoglobin in grams per 1000 c.c. of blood.

2. Make an erythrocyte count.

3. Divide the hemoglobin in grams per 1000 c.c. of blood by the millions of erythrocytes per cubic millimeter:

$$\frac{\text{hemoglobin in grams per 1000 c.c. of blood}}{\text{erythrocytes in millions per cubic millimeter}} = \frac{\text{Mean Corpuscular Hemoglobin}}{\text{(micromicrograms)}}$$

4. The normal range for adults is from 27 to 33. The range for children from 4 to 13 years of age is 20 to 28 (Osgood and Baker).

METHOD FOR DETERMINING THE MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION

Principle.—This is the mean or average hemoglobin concentration in gm. per 100 c.c. of packed cells. It has little clinical value. Rarely if ever are the cells found to be supersaturated and only in a few conditions such as chlorosis is there less than normal.

Procedure.—1. Determine the hemoglobin in grams per 100 c.c. of blood.

2. Determine the volume of packed cells in c.c. per 100 c.c. of blood.

3. Divide the grams of hemoglobin by the c.c. of packed cells and multiply by 100:

$$\frac{\text{hemoglobin in grams per 100 c.c. of blood}}{\text{volume of packed cells in c.c. per 100 c.c. of blood}} \times 100 = \begin{array}{c}\text{Mean Corpuscular}\\\text{Hemoglobin}\\\text{Concentration}\end{array}$$

4. The normal range is from 33 to 38 gms. per 100 c.c. of packed cells.

METHOD FOR DETERMINING THE SATURATION INDEX

Principle.—The same as the mean corpuscular hemoglobin concentration.

Procedure.—1. Determine the volume index.

2. Determine the color index.

3. Calculate the saturation index as follows:

$$\frac{\text{color index}}{\text{volume index}} = \text{Saturation Index}$$

4. The normal range is 0.90 to 1.2.

The chart shown in Figure 62 by Haden gives a classification of the anemias based on the number, volume and hemoglobin content of red blood corpuscles.

METHOD FOR COUNTING TOTAL LEUKOCYTES

Principles.—1. Blood is accurately diluted 1:20 with a fluid producing complete hemolysis of erythrocytes but without injury to the leukocytes. The corpuscles contained in 1 c.mm. of the diluted blood are then counted in a special chamber and this number multiplied by 20 (the dilution factor) to obtain the number in 1 c.mm. of undiluted blood. This constitutes the method for reporting the results.

2. The normal number of leukocytes is usually stated to be from 5000 to 10,000 per c.mm.; however, normal counts may be slightly lower or higher according to age, influence of digestion, *the time of day*, and other little understood factors. There is a greater normal fluctuation in the number of leukocytes than in the erythrocytes. In the morning the number is usually the lowest and gradually rises until evening, probably as a result of exercise. There may be as much as a 100 per cent increase but still the count may fall within the normal range. It is therefore advisable to *record the time of day a leukocyte count is made*. A count made during the afternoon may be 2000 higher than one made in the morning. Such an increase may not be necessarily due to disease.

Procedure.—1. Draw blood to the mark 0.5 of the Thoma pipet marked 11, or fill the stem of the Trenner pipet, as described in the method for counting erythrocytes.

2. Draw up diluting fluid to mark 11, thus making a dilution of 1:20.

DILUTING FLUID FOR LEUKOCYTES [4]

Acetic acid .... .3 to 5 c.c.

Water .... 100 c.c.

Add a few drops of an aqueous solution of gentian violet or methylene blue to slightly stain the leukocytes.

3. Rotate the pipet well for several minutes, holding it in a horizontal position; finally shake sideways.

4. Blow out several drops.

5. Fill the counting chamber in exactly the same manner as described for the counting of erythrocytes.

6. Allow the cells to settle for at least three minutes.

7. Center the light and focus exactly as described for the erythrocyte count.

8. If the Levy or Levy-Hausser chamber is used, the cells in the four corner sq. mm. areas are counted in both rulings of the double chamber, also the cells in both central sq. mm. areas, i.e., the cells in ten sq. mm. areas in all. This count is multiplied by 20 (dilution factor), which gives the total leukocytes per c.mm. of undiluted blood. *Or count the cells in the four corner squares and multiply by 50.*

9. When the cells are counted in one ruling only, the total count of the five sq. mm. areas, as above, will represent the number of leukocytes in 5 sq. mm. or 0.5 c.mm. and must be multiplied by 40.

10. If other rulings are used count the number of cells per sq. mm. (the entire ruled-off area) and multiply by 200.

11. The same errors as may occur in erythrocyte counting must be kept in mind and carefully avoided.

METHODS FOR STAINING BLOOD SMEARS

Principles.—1. An examination of the erythrocytes and different kinds of leukocytes possesses a great deal of diagnostic value. For this purpose stained preparations are superior to unstained or wet preparations.

2. The blood must be prepared in thin, evenly distributed smears on slides or cover glasses as described on page 56. *Well prepared films or smears are absolutely essential for accurate results.* For routine work a variety of staining methods are available. Preference should be given the polychrome stains, that is, those capable of staining the neutral, acid and basic granules of leukocytes. Poorly prepared and faultily stained smears cannot give results of acceptable accuracy.

[4] N/10 HCl can also be used as a diluent and after the leukocyte count is made the remaining fluid can be used for determining the hemoglobin by the Haden method.

Wright's Method.—1. Cover smear with 10 drops of Wright's stain [5] and allow to remain for one minute.

2. Add to the stain on the smear an equal amount of distilled water and allow the stain thus diluted to stand three minutes more (the stain should now have a "brassy" sheen).

3. Wash off sheen with tap water and stand on end to dry.

4. Dry and mount in balsam or gum damar if cover glass is used.

5. This is an excellent polychrome stain and is recommended for routine work.

6. The following method, employing a buffer solution, is also recommended by Giordano:

(*a*) Buffer solution: 6.63 grams of acid potassium phosphate (KH_2PO_4) and 3.2 grams of dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) are dissolved in 1000 c.c. of distilled water.

(*b*) Add 25 to 30 drops of Wright's stain to blood film and allow to stand one minute. Add an equal number of drops of buffer solution and allow to remain four minutes. Wash with distilled water and dry.

7. Feemster recommends the following method:

(*a*) Solution No. 1: Place enough dry stain in a clean dry bottle to saturate absolute methyl alcohol (not more than 0.3 gram will dissolve in 100 c.c.). Allow to stand for one or two days with occasional shaking. Filter and add one-fifth of its volume of methyl alcohol.

(*b*) Solution No. 2: In a second bottle place 90 c.c. of 95 per cent ethyl alcohol and 10 c.c. of distilled water. Add 0.3 gram of dry stain; mix and stand for two days with occasional shaking. Filter before use.

(*c*) Cover the dried blood smear with solution No. 1; *drain off excess at once* and let stand until the slide *turns red*.

(*d*) Flood with distilled water; let stand for one or two minutes.

(*e*) Wash with solution No. 2 until most of the red precipitate disappears; that over the smear usually disappears immediately. Wipe off ends of slide.

(*f*) Wash with distilled water, dry and examine.

Jenner's Method.—1. Cover smear with Jenner's stain for three to five minutes.

2. Wash carefully with water until lavender color.

3. Dry; mount in balsam or gum damar if cover glass is used.

4. This is a polychrome stain and generally satisfactory.

Giemsa's Method.—1. Fix the smear in methyl alcohol for two to five minutes.

2. Submerge the slide in diluted Giemsa's stain for twenty-five to thirty minutes:

Giemsa stain	1 c.c.
Water (distilled)	10 c.c.

The water should be neutral or very slightly alkaline. It is advisable to test it before use by placing a few grains of hematoxylin in about 5 c.c. In from one to five minutes a pale violet color should appear; if it does not, the water is unsuitable for use.

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[5] Wright's, Jenner's, and Giemsa's stains may be purchased from supply houses.

The stain should be used immediately after diluting.

3. Wash with distilled water and dry thoroughly.

Pappenheim's Pyronine-Methyl-Green Method.—1. Fix smears with heat.

2. Cover with the staining fluid for from one-half to five minutes.

> Methyl green (sat. aq. sol.) .... 30 to 40 c.c.
> Pyronine (sat. aq. sol.) .... 10 to 15 c.c.

This mixture will keep for about one month. If it is found that one of the dyes stains too intensely, it can be reduced by adding more of the other dye.

3. Wash with distilled water and thoroughly dry.

4. This stain is for special purposes only.

METHOD FOR SUPRAVITAL STAINING (DOAN)

Materials:

(a) Chemically clean, grease free slides

(b) Chemically clean, grease free No. 0 coverslips

(c) Supravital dyes (tested stains are available from National Aniline and Chemical Company; Eastman Kodak Company (Pinacyanole)

(d) Neutral absolute ethyl alcohol

Method of preparation of glassware:

(a) Place new glassware in cleaning solution (sat. $K_2Cr_2O_7$ in H_2SO_4) for 48 hours

(b) Wash in running tap water for 48 hours

(c) Rinse well with distilled water (3 times)

(d) Store in 80% ethyl alcohol

(e) Wipe dry with clean gauze; avoid lint

(f) Store in dust proof container

Method of preparation of solutions:

1. Stock solutions.

(a) Neutral absolute ethyl alcohol (distilled over CaO)

(b) Saturated solution of vital Neutral Red in neutral absolute alcohol

(c) Saturated solution of Janus Green (Diazine Green) in neutral absolute alcohol

(d) 0.1% solution of Pinacyanole in neutral absolute alcohol

2. Staining solution for blood (to be made up just before using)

(a) 5 c.c. neutral absolute alcohol

(b) 35 drops (Wright's capillary pipet) saturated alcoholic Neutral Red solution

(c) 5 drops (Wright's capillary pipet) saturated alcoholic Janus Green solution

3. Staining solution for blood where Pinacyanole is desirable as a mitochondrial stain (to be made up just before using). The mitochondria remain visible for a longer period than with Janus Green (Hetherington).

(a) 5 c.c. neutral absolute alcohol

(b) 20 drops (Wright's capillary pipet) saturated alcoholic Neutral Red solution

(c) 6 drops 0.1% Pinacyanole alcoholic solution

4. For leukemic blood, bone marrow and other more cellular tissues an increased concentration of dyes is necessary. proportional to the number of cells present reacting specifically with each dye.

Preparation of slides with stains:

Flame slides prepared as above, cool, flood with stain. drain excess back into bottle, and air-dry.

Store in dust proof container.

Procedure.—1. Take a small drop of fresh living material on a coverslip (blood, spinal fluid, fluid from the serous cavities, bone marrow, tissue scrapings or suspension).

2. Invert on stained slide; do not press from above or cells will be injured.

3. Seal edges of coverslip with any high melting point vaselin or paraffin.

4. Study in warm box or at room temperature; optimum for cellular motility 98.8° F.

CLASSIFICATION OF NORMAL LEUKOCYTES

1. Three separate kinds are recognized. namely, (a) lymphocytes from lymphoid tissues: (b) monocytes from the reticulo-endothelial system and (c) granulocytes from bone marrow.

2. The last are so called because of granules in their cytoplasm and are subdivided according to the staining reactions of these into three types: neutrophils, basophils and eosinophils.

3. *Lymphocytes* (Plate I) vary in size from about that of an erythrocyte to that of a neutrophil. The nucleus is round and stains deeply with the basic stain. The smaller ones stain more deeply and have a small amount of cytoplasm. The larger ones often stain less intensely and have more cytoplasm, in some of which may be seen several round, reddish-purple azurophilic granules. Occasionally forms with indented nucleus appear. It is generally believed that the large, less deeply staining forms are the younger types which become smaller upon reaching maturity. An increase of these cells is called *lymphocytosis* and a decrease *lymphopenia*. Normal 1000 to 3000 per c. mm.

4. *Monocytes* (Plate I) include cells which were formerly called large mononuclear leukocytes and transitionals. They are sometimes known as endotheliocytes. They are the largest type of leukocyte found in the blood (14 to 20 micra). The nucleus is less deeply stained than that of the lymphocytes, is usually indented and at times is horseshoe shaped. The chromatin material in the nucleus has a skeinlike appearance. Those with round nuclei are often difficult to distinguish from lymphocytes. There is a wider band of cytoplasm than that of the lymphocytes. The lymphocytes are not usually as large as neutrophils, while the monocytes are usually larger. The chromatin of lymphocytes is more granular in appearance. An increase is called *monocytosis*. Normal 100 to 600 per c. mm.

5. *Neutrophils* (Plate I) are easily recognized by an irregular-shaped and lobulated nucleus, for which reason they are commonly known as "polymorphonuclears." Their average size is about 12 micra. The nucleus may be ribbon, band-like or segmented. The segments vary in number from one to six or seven and are all connected by narrow nuclear bands. The cytoplasm contains numerous fine granules which do not stain definitely either blue (basic) or red (acid) and hence are regarded as neutral or neutrophilic. They may undergo an increase, designated as "neutrophilia," or a decrease, called "neutropenia." They have been subdivided by Arneth, Schilling, and others according to the number and shapes of nuclear segments (described below). Normal 3000 to 7000 per c. mm.

6. *Eosinophils* (Plate I) are granulocytes similar to the neutrophils except for a difference in the size and staining properties of the granules, which are round or oval and large enough to be distinctly outlined. They stain pink to bright red (acid stain) with Wright's stain. An increase is called "eosinophilia" and a decrease "eosinopenia." Normal 50 to 400 per c. mm.

7. *Basophils* (Plate I) are granulocytes similar to the neutrophils except that they contain granules which are larger and stain deep purple (basic stain) with Wright's stain. The nucleus is usually without distinct lobulation. The cell itself is slightly smaller than the neutrophil. They are also called "mast cells." An increase is called "basophilia" or "basophilic leukocytosis." Normal 0 to 50 per c. mm.

QUALITATIVE CHANGES IN NEUTROPHILIC LEUKOCYTES[a]

Shifts to the Left and Right.—Arneth in 1904 attempted to measure the relative age of the circulating neutrophilic leukocytes from the morphology of the nucleus. He showed that the nucleus of the neutrophil, in its development from the myelocyte in the marrow, becomes gradually more indented, and divides with age into an increasing number of separate lobes or segments. The shape of the nucleus is thus an index of the maturity of the cell. He divided the neutrophils into five major groups and numerous subgroups on the basis of nuclear configuration (Fig. 74). The nucleus in group I has only one lobe and in group V it has five or more lobes. Arneth suggested the terms "shift to the left" to indicate an increase in young cells or in those with fewer lobes, and "shift to the right" to designate an increase in older cells or in those with a larger number of lobes. Arneth's complete classification is unfortunately entirely too complicated for practical laboratory application. His observations on the relation of the nuclear form to the age of cell, however, have been accepted as fundamentally correct, and have stimulated further study and classification of the maturity of the neutrophils which have proved of great value in clinical hematology.

Schilling in 1911 suggested a classification of the neutrophilic leukocytes (Fig. 75) which has been widely employed especially in the study of diseases related to infection. He divides the neutrophils in the order of their age into four groups: (1) myelocyte; (2) juvenile nuclear in which the nucleus has become indented; (3) stab or staff nuclears in which the nucleus is T-, V- or U-shaped

[a] R. L. Haden. *Am. J. Clin. Path.* 1935, 5:354.

CLASS				
I *OneNucleus*	M 0.0%	W 0.2%	T 5.0%	
II *Two-Lobed Nuclei*	2K 0.27%	2S 23.5%	1K 1S 11.7%	
III *Three-Lobed Nuclei*	3K 23%	3S 56%	2K1S 16.7%	1K2S 16.4%
IV *Four-Lobed Nuclei*	4K 3.8%	4S 0.07%	3K1S 6.4%	3S1K 1.6%	2K2S 4.7%
V *Five-Lobed Nuclei*	5K 1.0%	4K1S 0.4%	3K2S 0.4%	4K2S 0.07%	3K3S 0.07%

M=Myelocyte W=Slightly Indented Nucleus
T=Deeply Indented Nucleus
K=Round Piece   S=Bent Piece

Fig. 74.—Arneth Classification of the Neutrophilic Leukocyte (Haden)

| A | B | C | D | E |
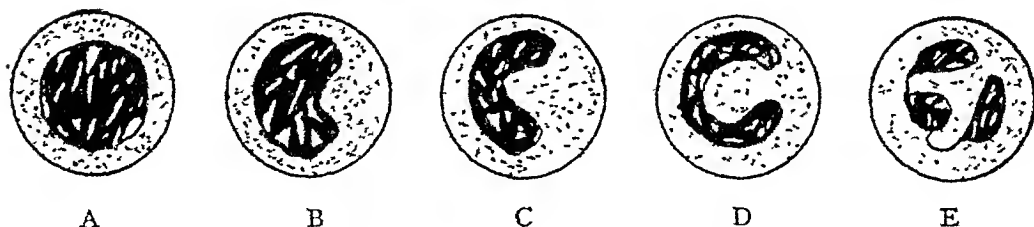
Fig. 75.—Schillings' Division of Neutrophils

A. A myelocyte (normal 0). *B*. A juvenile cell (normal 0 1 per cent). *C* and *D* stab cells (normal 3-5 per cent). *E*. segmented cell (normal 51-67 per cent).

without division into segments; and (4) segmented nuclears which are fully differentiated neutrophils with distinct segmentation into from two or five lobes. Schilling's classification is much simpler than Arneth's and recognizes two types of "shift to the left." Arneth, in his "shift to the left," considered that the increased number of cells with fewer lobes was caused only by the rapid outpouring of leukocytes from the marrow in response to an acute need before growth and differentiation were complete. Schilling designates this a *regenerative* "shift to the

Fic. 76.—Cooke and Ponder's Classification of the Neutrophilic Leukocyte (Hawes)

left." In some instances a depression of bone marrow function due to toxins seems to prevent the complete differentiation of the neutrophils, so that they develop only to a certain point and emerge into the circulation at this stage. The differential count in such cases shows an increased number of immature forms due to the depressed marrow function. Schilling designates this type of reaction as a *degenerative* "shift to the left." Other evidences of degeneration of the neutrophils are also seen here, such as loss of structure and narrowing and deep-staining of the nucleus, irregularity in size and staining reaction of the granules in the cytoplasm and the vacuoles.

Schilling in classifying the neutrophils, rightly places the emphasis on the more immature forms. Arneth had emphasized the subdivision of the segmented or more mature types. It is also often difficult to determine when the nucleus of any given cell has become segmented. Cooke and Ponder suggest a simple criterion, which is now known by their names, for determining division of the nucleus. The nucleus never divides completely. The lobes are connected either by a fine filament or by denser bridges of nuclear material. Cooke and Ponder do not classify a nucleus as divided if the segments are connected by other than a fine chromatin thread (Fig. 76). By using this criterion of division it is relatively simple to classify all neutrophils in a well-made film. Cooke and Ponder recognize five groups of neutrophils having from one to five distinct lobes in the nucleus. While this is a valuable simplification of Arneth's classification, the emphasis is placed on subdivision of the segmented or more mature cells as in

Arneth's method, rather than on the more immature cells. It is desired to know the degree of immaturity of the neutrophils rather than the extent of maturity. Pernicious anemia is one of the few clinical conditions in which the appearance of a larger number of very mature neutrophils or "shift to the right" is of diagnostic importance. Some "shift to the left" is, however, encountered in the presence of almost every infection, and often in other toxic conditions.

Several observers have simplified still further the grouping of the neutrophils and at the same time they have preserved the essential and valuable features of this procedure. Myelocytes are seen infrequently. Pons and Krumbhaar sugggest the division of the neutrophils into three classes: (1) metamyelocytes or very young forms in which there is only a slight indentation of the nucleus (the juvenile form of Schilling); (2) nonsegmented or young neutrophils (stab form of Schilling; and (3) segmented or older cells (Fig. 77). These authors do not mention criteria for differentiating segmented and nonsegmented cells although their illustrations show as segmented cells only those in which the lobes are connected by a filamentous thread. Farley. St. Clair and Reisinger suggest the use of Cooke and Ponder's criterion for determining segmentation and the simple division of all neutrophils into filamented and nonfilamented

Fig. 77.—Pons and Krumbhaar Classification of the Neutrophilic Leukocyte (Haden)

cells (Fig. 78). Neutrophils in which two or more lobes are united only by a filament of chromatin material are recorded as filamented cells. all others are classified as nonfilamented.

The classification of neutrophils into filamented and nonfilamented forms is simply, quickly and accurately made and usually supplies most of the valuable information concerning the degree of maturity of the neutrophil. Schilling's classification is valuable when a more detailed study of maturity is required. Arneth's complete classification has been discarded because it is entirely too complicated for practical use. Cooke and Ponder's count usually gives information of little more value than the simple division of all neutrophils into filamented and nonfilamented forms.

Procedure.—In both the Schilling hemogram and the filament-nonfilament count 100 or more leukocytes are counted and the neutrophils are subdivided. When 100 leukocytes of all types are counted, not more than 16 per cent of the neutrophils should be nonfilamented.

A normal average of neutrophils according to Schilling is found when 100

leukocytes of all types are counted as (1) myelocyte, none; (2) juvenile, 0-1%; (3) stab, 3 to 5%; and (4) segmented, 51 to 67%. In making any of the qualitative counts, a thin, well-stained film is necessary. It is far preferable to use only coverglass preparations. The films are stained routinely by the use of Wright's stain and a buffer solution.

Schilling suggested an index for expressing the degree of "shift to the left"
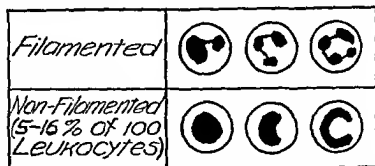
FIG. 78.—FARLEY et al. CLASSIFICATION OF THE NEUTROPHILIC LEUKOCYTE (HADEN)

which is obtained by dividing the immature forms (nonfilamented) by the mature (filamented) forms. It is expressed in the form of a fraction.

$$\text{Schilling Index} = \frac{\text{Per cent or number of immature neutrophils}}{\text{Per cent or number of mature neutrophils}}$$

Another method of expressing the shift is by dividing the mature (filamented) forms by the immature (nonfilamented) forms (nuclear index). This is the same as the Schilling index inverted and so expresses the index as a whole number.

$$\text{Inverted Schilling Index} = \frac{\text{Per cent or number of mature neutrophils}}{\text{Per cent or number of immature neutrophils}}$$

Either of these indices can be used to express the shift obtained with any of the classifications described. However, the normal limit will vary. Schilling gives as normal for his index any figure over 1/13 which corresponds to a nuclear index of 13. If the filament nonfilament classification of Farley et al. is used the normal will be higher for the Schilling index and lower for the nuclear index. When using the Schilling index the higher the number the greater the shift to the left and when the inverted index is used, the lower the number the greater the shift to the left.

Basophilic or "Toxic" Granulation in the Neutrophils.—More recently qualitative changes in the granules of the cytoplasm of the neutrophilic leukocytes have aroused much interest in relation to infection and other toxemias. It has been pointed out by numerous observers that deeply staining basophilic granules may occur in the neutrophils under abnormal conditions. The granules in the cytoplasm of normal neutrophils are numerous, small, of uniform size, and pinkish in color. The basophilic granules or "toxic granules," as they are often designated, may be large or small (Fig. 79). If the "toxic granules" are small, they are usually distributed among the pinkish neutrophilic granules; if they are large,

few if any normal granules are seen in the cytoplasm. The "toxic" granules occur especially in the presence of pneumonia, septicemia, and peritonitis but they may be found in any severe infection or toxemia with or without leukocytosis. The granules may be present in either young immature cells or in mature, segmented
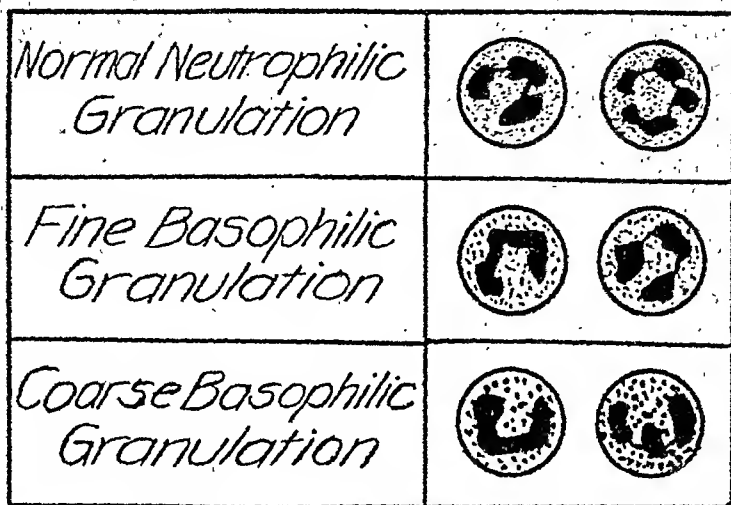
FIG. 79.—DIAGRAM TO INDICATE THE APPEARANCE OF BASOPHILIC OR "TOXIC" GRANULATION IN THE NEUTROPHILS (HADEN)

neutrophils. Large basophilic granules are found only in the acute stage of disease (Fig. 80).

In studying cells which show basophilic granulation, one is impressed with the small total number of normal granules in the cytoplasm. In a normal neutrophilic cell the cytoplasm is packed with small granules. If such a cell is stained by an oxidase method, the granules are sharply defined and the filling of the cell is evident. Likewise, an oxidase stain on a cell which shows basophilic granulations reveals relatively few oxidase reacting granules. Therefore, the oxidase stain also gives valuable information in the cytoplasm. Graham pointed out long ago a close relationship between an increase in the younger Arneth forms and a decrease in the number of oxidase reacting granules in the cytoplasm. He likewise showed that conditions such as lobar pneumonia, which is now recognized as a disease in which basophilic granulation is almost uniformly present,

FIG. 80.—DÖHLE INCLUSION BODIES × 1500. (Wood.)

exhibit also a great decrease in oxidase granules, although he did not mention basophilic granulation in this regard. He considered a decrease in the granules as evidence of the presence of a toxic condition. Basophilic granulation, then, really represents granule failure in the same manner as does a decrease in oxidase staining granules.

Basophilie or "toxic" granulation in the neutrophils is looked upon as a phenomenon of degeneration. It seems most probable that it represents the effect of the toxemia on the cells in the marrow at the stage of granule formation. The percentage of cells which show basophilic granulation may be recorded, or the degenerative index suggested by Kugel and Rosenthal and based on the percentage of neutrophils showing basophilic or "toxic" granules may be used. The index is calculated by dividing the number of neutrophils showing basophilic granulation by the total number of neutrophils counted.

$$\text{Degenerative Index} = \frac{\text{Number of neutrophils showing basophilic granulation}}{\text{Total number of neutrophils}}$$

In addition to changes in the granules, the staining reaction of the cytoplasm also often differs from that of normal. The cytoplasm may take on a bluish hue of varying intensity which is due to increased basophilia. This diffuse basophilic reaction together with "toxic" granulation occurs frequently. Likewise the pattern of the nucleus may exhibit abnormal variations in density of staining.

Basophilic granules and variations in the staining reaction of the cytoplasm and the nucleus may be seen satisfactorily only on thin films which are properly stained. If cover glass preparations are used, control preparations of normal blood should be stained and examined at the same time. If the blood film is made on a slide, a film of normal blood may be spread on one end of the slide and the two films stained at the same time. Variations in the unknown blood are significant only if they are absent from the normal film. It is important in studying all qualitative changes in the neutrophils to examine serial preparations because much more is learned from a comparison of counts made from day to day or time to time than is learned from single counts.

ABNORMAL VARIETIES OF LEUKOCYTES

The following leukocytes are not found in the circulating blood in health. Indeed the younger forms are even rare in the bone marrow except when activated by disease. At birth all the bones contain active bone marrow but in the adult in health only the flat bones such as the scapulae and the ribs and the bodies of the vertebrae are characterized by formative marrow. Cells of two series are encountered as follows:

Granulocytic Series.—1. *Myeloblasts.*—These are the youngest or stem cells of this series. They are slightly larger than the well known neutrophil leukocyte (18 to 20 microns) and possess a deep blue basophilic cytoplasm when stained with Wright's stain. There are no cytoplasmic granules. The nucleus is characterized by fine chromatin markings in the form of a fine stippling. To some observers the spaces between the chromatin markings suggest a fine sieve-like character. The nucleus stains bluish red by Wright's method. There are several small nucleoli which are stained light blue (Plate I).

2. *Premyelocytes.*—As the myeloblast matures, it acquires cytoplasmic granules. It is then called the premyelocyte. At first these granules are of the azurophile

variety, so called because with Wright's stain they take a rich blue stain. They are not affected by the oxidase stain of Goodpasture and although they are generally large granules, they vary much in size. The cytoplasm is less basophilic than in the myeloblast. The nucleus stains somewhat more deeply and the chromatin particles seem somewhat coarser. As this cell grows older, the azurophile granules are replaced by specific granules which become black when treated with Goodpasture's oxidase stain. It is at this point that the cell differentiates into one of the three following specific types of myelocytes: neutrophilic, eosinophilic or basophilic.

3. *Neutrophilic Myelocyte.*—The cytoplasm has at this stage reached its complete development and acquired phagocytic powers. The cytoplasm is very lightly acidophilic and packed with small, not easily seen, violet granules (Wright's stain). The cell is slightly smaller than the myeloblast (12-18 microns). The nucleus is still round but it is more deeply stained, its chromatin markings are coarser and nucleoli are rarely found. Further maturity of the cell is shown not by cytoplasmic changes but by changes in the nucleus. The round form becomes indented (juvenile leukocyte), the indentation extends deeper (stab leukocyte) and finally segmentation occurs (segmented leukocyte). During this process of indentation and segmentation, the chromatin condenses so that it stains more deeply from blue-red to bluish-black; the chromatin masses and nuclear membrane become thicker, and the spaces between the chromatin markings become wider (Plate I).

4. *Eosinophilic Myelocyte.*—This cell develops granules which are much larger than the neutrophilic granules and they take a distinct red, sometimes brownish-red stain (Wright's stain). The nuclear changes of this cell are similar to those of the neutrophile and the same type cells are formed (Plate I).

5. *Basophilic Myelocyte.*—In this cell the granules are also large like those of the eosinophil but they stain a deep blue although an occasional metachromatic (red or bluish-red) granule is encountered. Nuclear maturity occurs as in the other forms of myelocytes. In active bone marrow cells of varying maturity are seen from the most immature to the mature segmented form. They are best studied in films prepared by smearing the marrow on a slide and staining in the same manner as blood films (Plate I).

Lymphoid Series.—Cells of this series may be found in the bone marrow as well as in the circulating blood in leukemic states. Normally they are found in lymphoid tissue.

1. *Lymphoblast.*—This cell is identical with the myeloblast in appearance though some claim that there are minute differences. It may be found in the germinal centers of lymphnodes in health.

2. *Young Forms of Lymphocytes.*—These are characterized by delicately stained nuclei of a vesicular character. They do not contain nucleoli. They are usually large cells from 10 to 20 microns in diameter. The cytoplasm like that of the mature lymphocyte is lightly basophilic. These young lymphocytes are frequently incorrectly called monocytes because of their delicate nuclear structure. They are

found characteristically in infectious mononucleosis. Young lymphocytes may or may not possess azurophile granules.

3. *Türk's Irritation Forms.*—The true nature and significance of these cells is disputed. The nucleus stains deeply, is round with irregular markings and the cytoplasm is intensely basophilic.

METHOD FOR THE DIFFERENTIAL COUNTING OF LEUKOCYTES

Principles.—1. Differential leukocyte counts refer to the actual number or percentage of different kinds present.

2. The number of cells to be counted and classified should be determined by the total leukocyte count. In routine work the following is recommended:

> For total counts under 10,000, classify 100 cells.
> For total counts of 10 to 15,000, classify 200 cells.
> For total counts of 15 to 20,000, classify 300 cells.
> For total counts of 20 to 25,000, classify 400 cells.
> For total counts over 25,000, classify 500 cells.

3. It is usual to submit a report in terms of the per cent of each type of leukocyte in the count, but a much better plan is to report the actual number of each leukocyte per c.mm. of blood which may be calculated from the total leukocyte count. If, for example, the total leukocyte count is 12,500 with 77 per cent neutrophils, the actual number of these cells per c.mm. of blood would be 125×77 or 9625.

4. According to the percentage system, the normal for adults is approximately as follows (total count 5000 to 10,000):

	Per Cent		
Neutrophils	50	to	70
Basophils	0.5	to	1
Eosinophils	1	to	4
Lymphocytes	20	to	30
Monocytes	2	to	6

5. According to the actual number per c.mm. of blood, the normal for adults is approximately as follows:

	Per C.mm.		
Neutrophils	3000	to	7000
Basophils	0	to	50
Eosinophils	50	to	400
Lymphocytes	1000	to	3000
Monocytes	100	to	600

6. For children the figures are approximately as follows:

TABLE IV

Leukocyte	Three Months to Three Years	Three to Five Years	Over Five Years
Neutrophils	2000 to 7000	3000 to 8000	3000 to 7000
Basophils	0 to 50	0 to 50	0 to 50
Eosinophils	25 to 700	50 to 700	50 to 400
Lymphocytes	4000 to 9000	2500 to 6000	1000 to 3000
Monocytes	25 to 700	25 to 700	100 to 600

7. As the percentages do not indicate the actual number, it is advisable and recommended to convert them into the numbers of each type per c.mm. of blood, although the medical profession as a whole is not yet acquainted with the normal figures given above with which to interpret reports. *The per cent of any type of cell may be increased or decreased without any change in the actual number* by a change in the total leukocyte count.

8. When the per cent of a cell is higher than normal without any indication of an absolute increase as determined by the total count, it is spoken of as a "relative increase." But this term, which is so generally used, should be abandoned as it does not properly express any definite change. For example, a "relative lymphocytosis" may be due to a diminution in the number of neutrophils without any change in the normal number of lymphocytes; or the number of lymphocytes may be actually increased.

9. For simplicity the following nomenclature is advocated:

Leukopenia: decrease in total leukocytes
Neutropenia: decrease in polymorphonuclears
Basopenia: decrease in basophils
Eosinopenia: decrease in eosinophils
Lymphopenia: decrease in lymphocytes
Monopenia: decrease in monocytes
Granulopenia: decrease in all granulocytic leukocytes
Leukocytosis: Increase in total number of leukocytes
Neutrophilia: increase in polymorphonuclears
Basophilia: increase in basophils
Eosinophilia: increase in eosinophils
Lymphocytosis: increase in lymphocytes
Monocytosis: increase in monocytes

Table V gives the usual changes in the leukocytes in disease.
Procedure.—1. Prepare several blood smears as described on page 56.
2. Stain with Wright's or other suitable stain (see page 76).

TABLE

Usual Changes in

Neutrophilia	Neutropenia	Lymphocytosis
Severe acute infections, particularly those due to cocci	Subacute and chronic infections	Convalescence from acute coccus infections
Acute loss of blood (especially hemorrhage into body cavities)	Common in diseases due to bacilli	Common to bacillary infections
		Syphilis
	Splenic anemia	Measles
Infarction	Agranulocytosis (malignant leukopenia)	Lymphatic leukemia
Myelogenous leukemia	Convalescence from severe acute infections	Chronic infections Infectious mononucleosis

3. Examine with low-power lens to determine if the leukocytes are well distributed. Look particularly at the edges and end of the smear. If they are not properly distributed, examine another smear.

4. If the slide proves satisfactory, systematically examine with oil-immersion lens by recording each type of leukocyte seen as the slide is moved from one field to another. The Marbel blood cell calculator (Fig. 41) is very convenient.

5. At the same time a special differential count may be made of the neutrophils for the forms of metamyelocytes to ascertain if there is any "shift to the left."

6. The red blood corpuscles should also be examined and any abnormalities noted with special reference to the number of nucleated cells seen during the count.

7. At least three separate parts of the slide should be examined in order to get an accurate result.

8. The normal differential leukocyte count for the adult is approximately as follows:

    Lymphocytes .... 1000 to 3000 (20 to 30%)
    Monocytes .... 100 to 600 (2 to 6%)
    Neutrophils (nonfilamented) . 150 to 500 (3 to 6%)
    Neutrophils (filamented) ... 2550 to 5350 (50 to 67%)
    Eosinophils .... 50 to 400 (1 to 4%)
    Basophils .... 0 to 50 (0 to 1%)

9. As an example of a "shift to the left" or an increase of metamyelocytes (non filamented neutrophils) the following count from a case of acute suppurative appendicitis in an adult with a total leukocyte count of 15,000 may be given:

V
LEUKOCYTES IN DISEASE

Lymphopenia	Eosinophilia	Eosinopenia	Monocytosis
Severe acute infections, particularly those due to cocci	Convalescence from acute infections, particularly those due to cocci	Severe acute infections, particularly those due to cocci	
Common in agranulocytosis (malignant leukopenia)	Parasitic diseases Hay fever Eczema		Typhoid fever
	Bronchial asthma		Hodgkin's disease
	Eosinophilic leukemia		Protozoan diseases
	Scarlet fever		Monocytic leukemia

Lymphocytes .... 1800 (12 %)
Monocytes .... 600 (4 %)
Neutrophils (nonfilamented) .... 2100 (14 %)
Neutrophils (filamented) 10,425 (69.5%)
Eosinophils 0
Basophils 75 (0.5%)

PEROXIDASE REACTION

Principles.—This test is employed for the identification of myelocytes in the differential diagnosis of the leukemias. It is based upon the principle that a study of the oxidizing ferments of leukocytes aids in differentiating those of myeloid origin (myelocytes) from those of other lineage. Owing to difficulties in staining, a large number of methods have been advocated.

Washburn's Method.—1. Thin smears should be made, allowed to dry and stained within three to four hours.

2. Flood the smear with 10 drops of solution No. 1 and allow to stand for one to one and one-half minutes.

SOLUTION No. 1

Benzidine base .... 0.3 gm.
Basic fuchsin .... 0.3 gm.
Sodium nitroprusside (sat. aq. sol.) .... 1.00 c.c.
Ethyl alcohol (95 per cent) .... 100 c.c.

Dissolve the benzidine and fuchsin in the alcohol in order named. Then add the nitroprusside solution. A slight precipitate may form at the bottom of the flask but does not interfere with the staining qualities. This solution will keep for eight to ten months.

3. Add 5 drops of solution No. 2 without pouring off No. 1 and allow to stand three to four minutes.

SOLUTION No. 2

Hydrogen peroxide .... 0.3 c.c.
Tap water .... 25 c.c.

This solution will keep for about two days.

4. Wash thoroughly with tap water (one-half to one minute).

5. While still wet, flood with 95 per cent ethyl alcohol and allow to stand three to four minutes, or until completely decolorized (i.e., when there is no more pink visible to the naked eye).

6. Wash thoroughly with tap water and dry.

7. Flood with Wright's stain and allow to stand for 2 to 3 minutes.

8. Add 14 drops of tap water (one and one-half times as much water as Wright's stain) and allow to stand for 20 to 45 minutes. Most normal and many abnormal bloods will stain well in 20 to 25 minutes but certain abnormal bloods, particularly the leukemic bloods, require 35 to 40 minutes.

9. Wash briefly with tap water, flood with 95 per cent alcohol for 3 to 5 seconds and immediately wash with tap water for 10 to 15 seconds.

10. Dry and examine.

The *normal polymorphonuclear neutrophil* is so full of large black peroxidase granules that frequently the characteristic pleomorphic purple nucleus is the only other element visible. In the few polymorphonuclears which show a smaller number of black granules, the cytoplasm may be made out as finely granular and either neutrophilic or slightly pinkish. In certain abnormal bloods, notably in the myelogenous leukemias, some of these cells may have very few or no peroxidase granules. The cytoplasm of these cells frequently appears to be vacuolated, suggesting that they are degenerated forms in which the substance producing the peroxidase reaction has already disappeared.

The nuclei of *eosinophils* stain the usual purple, as with Wright's stain, and the large eosinophilic granules take on the deep black of the peroxidase stain but remain refractile. This gives them the appearance of very large black granules whose center is slightly paler than the periphery and of a brownish tint.

The *basophils* are the only normal cells which are hard to classify with this stain, since the basophilic granules take on the black of the peroxidase reaction so that their differentiation from neutrophils is difficult. The granules are slightly larger and tend to be arranged more thickly at the cell edges. The nucleus is also to be less distinguished as less pleomorphic and of a paler purple or even lavender color compared with that of the neutrophils.

The *lymphocytes* never show any peroxidase granules. Their characteristics by this staining method are identical with those seen in a Wright stain.

The *monocyte* (endothelial type of mononuclear cell) usually shows a scattering of black granules which tend to be present largely in groups. The remainder of the cell stains as with Wright's stain. That is, the nucleus is a slightly paler shade of purple than that of the neutrophils or lymphocytes while the cytoplasm shows a slate-gray homogeneous background with fine pinkish granulations unevenly distributed. Occasionally cells with only one or two or even without any peroxidase granules are seen.

Myelocytes usually show some peroxidase granules. The number may vary from 2 to 3 to a score or more, so that the cell is as full of granules as a mature neutrophil. The myelocytes which are less granular by Wright's stain usually show fewer peroxidase granules.

Myeloblasts and lymphoblasts never show any peroxidase granules so that their differentiation is no more possible with this stain than with any other, if a difference actually exists. Their appearance is identical with that seen in Wright's stain. However, it is worth remembering that some of the large primitive leukocytes with deep blue cytoplasms and large round purple nuclei may be very early myelocytes whose granules are too few and small to be seen with the ordinary Wright's stain. Such cells will show a few black granules with the peroxidase stain, thus indicating a myelogeneous origin.

NORMAL AND ABNORMAL ERYTHROCYTES

1. The erythrocytes of stained blood smears should also be examined and reported upon, especially in the anemias.

2. Normally they are about 7.5 micra in diameter (Plates I and II). In stained preparations distorted shapes may be seen due to mechanical distortion in preparing smears. In wet preparations they may occur in rouleaux formation and show some crenation.

3. In the anemias with diminished hemoglobin, and especially in chlorosis, the central pale area becomes larger and paler, constituting *achromia* (Plate I). In extreme instances the cells become mere rings ("pessary forms"). In pernicious anemia, however, many of the corpuscles stain deeply and entirely lack the pale center.

4. Abnormal variation in size is called *anisocytosis* (Plate II). When smaller than normal the cells are called *microcytes* (Plate II) and when larger, *macrocytes* (Plate II); extremely large forms are called *megalocytes*. In congenital hemolytic jaundice the erythrocytes are decreased in diameter but increased in thickness appearing as deeply stained cells (called "spherocytes").

5. *Poikilocytes* are corpuscles with abnormal shapes (Plate I). They may be caudate, club-shaped, oval, elliptical or sickle-shaped.

6. *Sickle-shaped* cells are especially numerous in a hereditary type of anemia more commonly seen in Negroes called "sickle-celled anemia" (Fig. 81). The Beck and Hertz method for examination is recommended (p. 95).

PLATE II.—NORMAL AND ABNORMAL ERYTHROCYTES.

The preparations are all from cases of erythremia (polycythemia vera) except No. 7 which is from a normal person. The stain is Wright's stain and the magnification is about 970. The drawings are made with the aid of a camera lucida and represent actual fields.

Group 1. Shows especially macrocytosis. The nucleated red cell is an erythroblast.

Group 2. Shows faint polychromatophilia and moderate anisocytosis. The flattening out of the cells is due to the increased viscosity of the polycythemic blood. Red cell count 10,000,000 per c.mm.

Group 3. Shows marked achromia, anisocytosis and polychromatophilia. The red cell count was 8,500,000 per c.mm. and the hemoglobin 105 per cent. Color index 0.6.

Group 4. Shows marked anisocytosis, poikilocytosis and polychromatophilia. The white corpuscle shown is presumably a myeloblast. The nucleus contains several nucleoli. Below this cell is a red corpuscle containing a Howell Jolly body. Red cell count 3,500,000 per c.mm.

Group 5. Shows numerous fragmented cells, macrocytes, macrocytes and coarse stippling. One of the red cells shows a Cabot-ring body. A normoblast is shown.

Group 6. Shows slight anisocytosis and fine stippling. The uniform gray color as compared with the cells of group 7 is due to a difference in the stain. In judging polychromatophilia the variation of the staining of individual cells in a given preparation and not the color of the preparation as a whole must be taken as a criterion.

Group 7. Shows normal red cells.

Note that in all the groups the number of platelets is normal or increased. (From Minot and Buckman, Am. J. Med. Sc., 1923, 166: 470.)

Pathological   *Normal*

Myelocytes.

Myeloblast

Eosinophil
myelocyte

1: Basophil
   myelocyte
2. Degenerated
   leucocyte
   (normal)

Poikilocytes

1. Hyperchromic
   macrocyte
2. Hypochromic
   cell
3. Microcyte

1. Megaloblasts
2. Normoblast

Basophilic
stippling

Malaria:
1. Benign tertiary
2. Estivo-autum-
   nal (tropical)

Young polymor-
phonuclear
(non-segmented
or non-filament
nucleus)

Mature polymor-
phonuclear
(segmented or
filament nu-
cleus)

Eosinophil poly-
morphonuclear

Basophil poly-
morphonuclear

Monocytes

Lymphocytes

Red cells
(erythrocytes)

Reticulated red
cells (reticulo-
cytes); 1 per
cent (cresyl
blue stain)

Clumped blood
platelets

0   10   20
Micra

PLATE I.—Normal and pathological blood cells. Wright's stain. ×1000. (Nicholson Laboratory Medicine.)

PLATE II
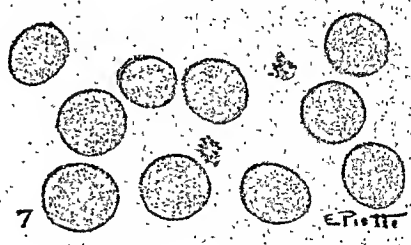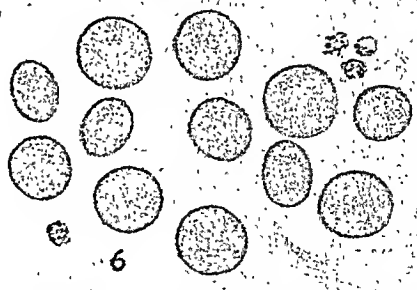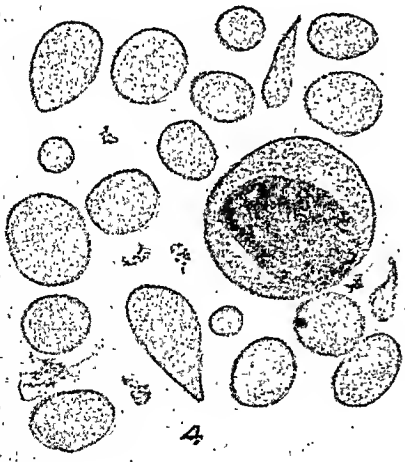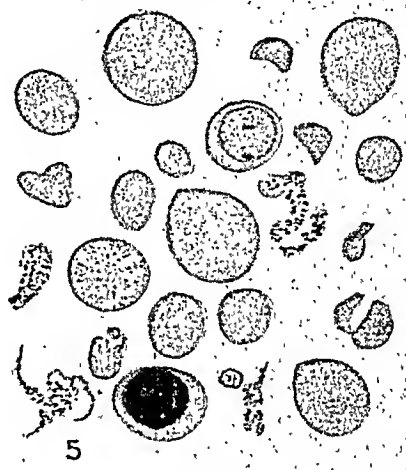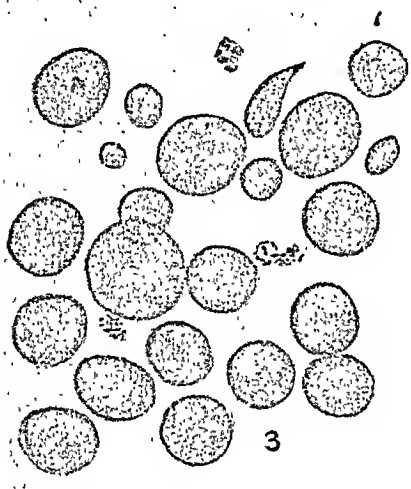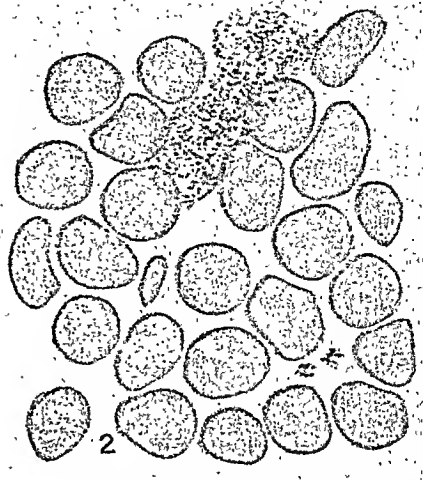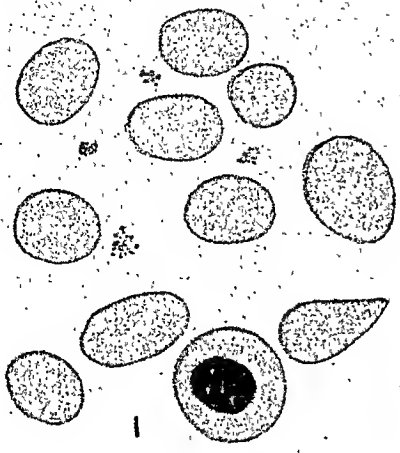
E.Piotti

Elliptically shaped cells may occur in both races in various anemias also as a hereditary phenomenon. The above test is a useful aid in differentiation and in the diagnosis of sickle-cell anemia.

7. *Polychromatophilia* is the term used for indicating the abnormal affinity of erythrocytes for the basic stains (Plate II). When present, many erythrocytes will be seen taking the basic blue stain in varying degrees, usually pale to light blue instead of pale pink. The condition is abnormal. and found in anemias where there is active regeneration of erythrocytes.

8. *Basophilic degeneration*, or *"stippling,"* is a condition in which there are many very fine to coarse blue dots or granules present in the erythrocyte (Plate II). They are found in cases where erythrocytic regeneration is active and probably represent cells which have undergone a degenerative change before they were fully mature. Their presence in suspected cases of lead poisoning is of diagnostic value.

9. *Reticulated erythrocytes* are young cells which, when stained with brilliant cresyl blue. show filaments which are well stained if the staining is done while the cell is still alive (vital staining). Although these filaments take the basic stain they will not stain in the usual dry smear. They are often arranged in skeins or wreaths (Plate II).

FIG. 81.—THE ERYTHROCYTES IN SICKLE-CELL ANEMIA

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B Saunders Co.)

10. *Nucleated red cells* are immature cells which are thrown into the circulation in severe anemias and leukemias in which there is an active regeneration of bone marrow. The *megaloblast* (Plate II) is the largest type and has a large, oval, pale-staining nucleus. The cytoplasm often shows polychromatophilia. Some may closely resemble lymphocytes due to the blue staining of the cytoplasm. These cells are usually present in pernicious anemia. where their presence is of diagnostic value. The *normoblast* (Plate I) is of about the same size as an erythrocyte and has a nucleus more deeply stained than the megaloblast. Occasionally the chromatin is arranged in a manner resembling the spokes of a wheel. They often show polychromatophilia. The older forms are smaller and the nucleus deeply stained. There may be more than one nucleus or the nucleus may be irregular, lobulated or fragmented. If the nucleus is completely broken up the fragments may all disappear except for a few. These remaining particles are called *nuclear particles* or *Howell-Jolly bodies*. The smallest nucleated red is called a *microblast*. It measures less than 5 micra in diameter. has a deeply staining nucleus and is regarded as an older form of normoblast.

Cabot's ring bodies are ring or figure-eight-shaped structures which stain red or reddish-purple with Wright's stain. They are seen in lead poisoning, pernicious anemia, leukemia and especially in erythroblastic anemia of children.

METHOD FOR THE PRODUCTION AND DEMONSTRATION OF SICKLE CELLS (BECK AND HERTZ) [7]

1. Place from 0.2 to 0.5 c.c. of saline citrate solution (3% sodium citrate in normal saline) in a test tube.

2. Clean and prick the finger as in collecting blood for a count.

3. Collect one or two drops of blood in the tube containing the saline citrate. Invert and mix.

4. Cover with sufficient oil to make a layer 1 cm. thick. Make sure no bubbles of air are under the oil.

5. Let the preparation stand at room temperature for twenty-four hours; then introduce 0.2 to 0.5 c.c. of formalin solution (0.05 gm. of sodium chloride to 1000 c.c. of 10 per cent neutral formalin) beneath the oil layer with a pipet. Thoroughly mix by forcing the liquids in and out of the pipet several times. Do not break up the oil layer for fear of letting air in too soon. Two or three minutes or more should be allowed for fixation. After this period the suspension is mixed again with the pipet to insure a uniform distribution of cells.

6. Remove a few drops from the tube, wipe away the excess oil from the tip, and place a drop on a glass slide.

7. Cover and examine.

8. The percentage is calculated in the manner of the differential leukocyte count.

The saline-citrate, the paraffin oil, and the formalin are to be kept in separate bottles, stoppered and labeled. One pipet is to be used for handling the saline citrate solution, one for the paraffin oil, and one for the formalin. Mark these pipets appropriately because a trace of formalin in the blood prior to sickling may prevent the deformity.

Permanent preparations can be made by making smears; air dry and fix in flame. Stain with 1 per cent aqueous solution of fuchsin. Formalin treated cells do not stain well with Wright's stain.

METHODS FOR DETERMINING THE DIAMETER OF ERYTHROCYTES

Method of Price and Jones.—1. Prepare smears in same manner as for differential counting. The films should be thin—the thinner the better—dry in air without heat.

2. Stain with Jenner's stain for 2 minutes, and after washing with distilled water and drying, superstain with weak aqueous solution of eosin for 2 minutes. These details should always be adhered to since it is found that alterations in the fixing and staining reagents can produce changes in the mean diameters of the cells.

3. Adjust some simple form of projection apparatus for a magnification of 1000 diameters and project the microscopic field onto a sheet of paper lying on the table. Outline in pencil the red cells. Two diameters, maximum and minimum, of each cell is then measured to 0.5 mm. with a glass millimeter scale and

can be directly expressed in terms of microns; the mean of these two measurements is accepted as the diameter value of the cell. The mean diameter of several hundred cells should be taken to represent the mean diameter for any sample of blood. According to Price-Jones the normal range is from 4.75 to 9.5 microns.

METHOD FOR COUNTING RETICULOCYTES

1. Place in a small test tube the following solutions and thoroughly mix:

Brilliant cresyl blue (Grubler) (sat. alc. sol.) .... 5 drops
Neutral potassium oxalate (1 per cent) in sodium chloride
   solution (0.85 per cent sol.) .... 25 drops

2. Prick the finger and allow 2 or 3 drops of blood to fall into the stain.
3. Mix thoroughly and allow to stand thirty minutes.
4. Centrifuge; pour off the supernatant fluid; transfer a small drop of sediment to a glass slide and make a thin smear. Allow to dry without heat. The sediment of cells should be of about the same density as the blood. To obtain this, leave about as much by volume of the supernatant fluid as cells. Mix the cells thoroughly before smearing and make thin films so they will dry rapidly.
5. Stain with Wright's or other suitable stain.
6. Estimate the per cent of red cells that are reticulated. This can be done by examining with the oil-immersion lens and counting 2000 cells, noting the number of cells showing reticulum.

$$\frac{\text{number of reticulated cells}}{20} = \text{per cent}$$

Normal findings are from 0.1 to 1.5 per cent. An increase is considered one of the early signs of regeneration of red cells.

Direct Smear Method.—1. Place a small drop of a solution of brilliant cresyl blue on the end of a slide and allow it to dry. Many slides can be made and kept on hand for further use.
2. Prick the finger or ear and place a drop of blood on the dye at one end of the slide.
3. Mix the blood with the dye with a match stick or glass rod and allow to stand about 30 seconds.
4. Make smear in same manner as for differential count.
5. Stain with Wright's or other suitable stain.
for the above method.
Slides or coverglasses may be painted with 0.5% alcoholic solution of brilliant cresyl blue and dried. Blood films are then made in the usual way, using these slides or coverglasses and counterstained with Wright's stain.

METHODS FOR COUNTING PLATELETS

Principles.—1. Blood platelets are stained by the polychrome dyes like those of Wright and Jenner and are spheric or ovoid, reddish to violet, granular bodies appearing as clumps in ordinary blood smears (Plate II).

2. Because of clumping, special methods for counting have been devised; divided into those that compute their number in relation to the number of erythrocytes (indirect) and those that make a direct count with a diluting pipet.

3. The exact source of platelets is as yet unknown, but it is generally believed that they are detached portions of the cytoplasm of megakaryocytes of the bone marrow (J. H. Wright) and have an important rôle in the coagulation of the blood.

Fonio's Smear Method.—1. Prepare and puncture finger with lancet and immediately place a drop of 14 per cent magnesium sulphate over the puncture before the blood begins to flow.

2. With gentle pressure allow the blood to flow into the sulphate solution. When the proportion is about 1 of blood to 5 of sulphate, mix thoroughly.

3. Transfer a drop to a clean slide and make a thin smear in same manner as described for differential counts. Prepare several smears.

4. Wipe the finger clean and proceed to make a red cell count.

5. Stain the slide with Wright's blood stain, being sure that the stain is of proper reaction to stain the platelets well as otherwise they may be palely stained and difficult to count.

6. Cut a small square in a circular piece of paper and place it in the ocular of the microscope to reduce the size of the field.

7. Focus and count the number of erythrocytes and the number of platelets in the field. Continue to count fields over various parts of the slide (center and both ends) until 1000 erythrocytes have been counted.

8. The number of platelets counted to 1000 erythrocytes is multiplied by the number of thousands of erythrocytes as determined by the erythrocyte count.

Example: Patient's erythrocyte count is 4,500,000. The number of platelets counted to 1000 erythrocytes is 39. Dividing the total erythrocyte count by 1000 to determine the number of thousands of erythrocytes gives 4500. Multiplying 39 by 4500 gives 175,500 as the platelet count.

$$\text{number of platelets counted} \times \frac{\text{erythrocyte count}}{1000} = \text{platelet count}$$

9. The normal is from 200,000 to 500,000 per c.mm. of blood.

Direct Method.—1. Rapid work is necessary in order to prevent clumping of platelets.

2. With a red-corpuscle pipet draw one of the diluting fluids given below to near the 1 mark; then blood from a freely flowing puncture to exactly 0.5 and finally diluting fluid again to 101 (blood dilution of 1 : 200).

Wright and Kinnicutt's Diluting Fluid

Brilliant cresyl blue (aq. sol. 1:300) ..... 2 parts
Potassium cyanide (aq. sol. 1:1400) ...... 3 parts

Keep the solutions separately. The dye keeps indefinitely; the cyanide solution keeps about ten days. Mix and filter just before using.

Rees and Ecker's Diluting Fluid

Sodium citrate (3.8 per cent aq. sol.) .... 100.0 c.c.
Formalin .... 0.2 c.c.
Brilliant cresyl blue.................... 0.1 gm.

Filter before using.

Leake and Guy's Diluting Fluid

Water (distilled) .... 94.0 c.c.
Formalin .... 6.0 c.c.
Sodium oxalate .... 1.6 gm.
Crystal violet .... 0.05 gm.

The fluid is warmed, filtered and kept in a bottle (keeps well).

3. Shake for 2 minutes.
4. Fill counting chamber as in making an erythrocyte count.
5. Allow to stand for 10 minutes.
6. Examine with 10X ocular and 4 millimeter objective.
7. Count the platelets in 200 small squares and multiply by 4000 to obtain the number per c.mm. of blood.

METHOD FOR DETERMINING THE COAGULATION TIME

Principles.—1. When the coagulation time of the blood is determined by methods using finger puncture to procure the blood, the results are only approximately correct and not entirely dependable, because of the admixture of tissue juices with the blood.

2. The coagulation time is prolonged in hemophilia. A much less impressive and uncertain prolongation is sometimes found in melena neonatorum, obstructive jaundice, some anemias and leukemias and some of the infectious diseases.

Capillary Tube Method.—1. Cleanse a finger and puncture as for blood count.

2. Fill a capillary glass tube (1.5 millimeters in diameter and 3 to 5 centimeters long) with blood. The tube will fill readily by capillary attraction if one end touches the drop of blood and the tube is inclined downward. *Note the time.*

3. At half-minute intervals after an interval of three minutes carefully break a small piece off the end of the tube, holding it in such a manner that the broken

ends are kept together; then separate the ends slowly and note if fibrin threads span between the ends. When the threads are seen to spread a distance of 5 millimeters or more, *note the time*.

4. *The time between the filling of the tube and the appearance of fibrin threads is the coagulation time.* The normal is from four to eight minutes with an average of about six.

Drop Method.—1. Cleanse and puncture finger as for blood count (puncture deep to insure free flow of blood).

2. Place several drops on a clean slide (the drops should be about 4 or 5 millimeters in diameter). *Note the time.*

3. At half-minute intervals draw a needle through one of the drops. As soon as the needle picks up fibrin threads and drags them along, coagulation has taken place. *Note the time.*

4. The time interval between placing the drop on the slide and the formation of fibrin shreds is the *coagulation time*. *The normal time is between two and eight minutes.*

Venous Puncture Method (Lee and White).—1. With a small syringe, fitted with a gage 20 needle, puncture a vein at the elbow and collect 1 c.c. of blood without using suction. *Note the time.*

2. Remove the needle from the syringe and place the blood in a test tube having a diameter of 8 millimeters. The test tube should be absolutely clean and rinsed with physiological salt solution just before the blood is placed in it.

3. Set the tube upright in a rack at room temperature or better in a water bath or glass of water at a temperature of 75° F.

4. At one-minute intervals tilt the tube to see if the blood still flows. As soon as it fails to flow and can be inverted, coagulation has taken place.

5. The interval between the time the blood is removed from the vein and the time the tube can be inverted without disturbing the clot is the *coagulation time*. The normal time is from five to ten minutes.

6. A control test is advised with the blood of a normal person.

METHOD FOR DETERMINING THE BLEEDING TIME

Principles.—1. This test is useful in the diagnosis of hemorrhagic diseases and with the coagulation time as a preoperative test.

2. The normal bleeding time is from one to three minutes. It is prolonged in cases where the blood platelets are markedly reduced and in chloroform and phosphorus poisoning; also in liver diseases which show a tendency to hemorrhage. In hemophilia the bleeding time is normal.

3. It is dependent upon the mechanical and probably the chemical action of the blood platelets; also upon the elasticity of the skin and the ability of the tissue juices to promote clotting.

Procedure.—1. Puncture the lobe of the ear or the finger so that the blood flows drop by drop without any assistance.

2. Note the time the first drop appears.

3. Remove with filter paper each drop as it forms, care being taken not to touch the skin.

4. Note the time bleeding stops.

5. The time interval between the appearance of the first drop and the removal of the last represents the bleeding time. Normally it is one to three minutes.

METHOD FOR DETERMINING THE PROTHROMBIN TIME

This test is useful in the diagnosis of hemophilia. The prolonged *prothrombin time* in this condition is probably due to the failure of the platelets to disintegrate and release thromboplastic material.

Howell's Method.—1. Secure 2 c.c. of blood by venous puncture in a syringe which has just been washed with physiological salt solution and without using suction.

2. Immediately place in a test tube containing 0.25 c.c. of a 1 per cent solution of oxalate in physiological saline solution.

3. Mix thoroughly by inverting and centifruge.

4. Remove the clear plasma and place 5 drops in each of four small test tubes.

5. Add 0.5 per cent calcium chloride as follows:

Tube 1 .... 2 drops
Tube 2 .... 3 drops
Tube 3 .... 4 drops
Tube 4 .... 5 drops

6. Note the time.

7. Mix gently and observe coagulation in the same manner as described above under venous puncture method for coagulation time. When a tube can be inverted without disturbing the clot, coagulation is complete. Note the coagulation time of the tube which coagulates first. This is the *prothrombin time*. Blood from a normal person should be tested at the same time as a control and for determining the *prothrombin quotient* of Jurwitz and Lucas:

$$\frac{\text{prothrombin time of unknown}}{\text{prothrombin time of normal}} = \text{prothrombin quotient}$$

8. The normal prothrombin time is about ten minutes.

9. The normal prothrombin quotient is about 1 and in hemophilia from 5 to 25.

METHOD FOR DETERMINING THE CALCIUM TIME

This test is used for determining if, in cases of prolonged coagulation time, the delay in coagulation is due to a deficiency of calcium.

Procedure.—1. Secure 2 or 3 c.c. of blood by venous puncture.

2. Place 1 c.c. of blood in each of two test tubes having a diameter of 8 to 10 millimeters.

3. To one of the tubes add 3 drops of a 1 per cent solution of calcium chloride.

4. Observe coagulation. If the tube containing the calcium coagulates within the normal time and the tube without calcium shows delayed coagulation, the

prolonged coagulation time of the blood is considered as being due to a deficiency in calcium.

METHOD FOR DETERMINING THE CLOT RETRACTION TIME

After coagulation has taken place the clot will contract and express serum. This is called retraction and the phenomenon appears to have some relation to the platelets. If the platelets are present in normal number, retraction occurs; if the platelets are greatly diminished, retraction will be retarded or absent. The test has no relation to the coagulation time even in hemophilia where the retraction is normal.

Procedure.—1. Secure 2 or 3 c.c. of blood by venous puncture.

2. Place in test tube and incubate at 37° C.; observe occasionally for a period of a day or two.

3. The first evidence of retraction is the separation of the clot from the wall of the tube and then the gradual expression of serum. Normally retraction is completed in from eighteen to twenty-four hours. In thrombocytopenic purpura it may be retarded or absent.

METHODS FOR DETERMINING THE SEDIMENTATION RATE OF ERYTHROCYTES

Principles.—1. It has been known for centuries that in some acute infectious diseases the erythrocytes may become separated from the plasma even before coagulation has occurred. During recent years a number of methods have been devised for determining the sedimentation rate of these cells, especially in pregnancy, tuberculosis, and acute bacterial infections.

2. An increase or change in the electrical charge of the erythrocytes, an increased cholesterol content, changes in viscosity of the plasma, variations in the erythrocyte count and certain chemical changes in the blood have been ascribed as causative factors, but none have been definitely proven or accepted.

FIG. 82.—CUTLER
BLOOD COLLECTING
TUBE

Collecting and Adjusting Blood Sample.—1. Cleanliness and dryness of apparatus are essential.

2. Place 1 drop of a saturated solution of potassium oxalate in a dry, clean tube (Fig. 82). This will prevent the coagulation of 8 to 10 c.c. of blood. Heller and Paul recommend 4 mg. solid potassium oxalate and 6 mg. solid ammonium oxalate for 5 c.c. of blood.

3. Obtain by venous puncture 5 to 10 c.c. of blood and place in tube containing anticoagulant, the amount depending upon the type of tube selected for performing the test.

4. Mix gently by inverting the tube several times.

5. Make an erythrocyte count or determine the volume of packed cells. If the count is between 4.5 and 5.5 million or the volume between 45 and 55 per cent, the specimen is suitable for testing. Otherwise, the erythrocyte concentration should be corrected as follows:

Method for correcting erythrocyte concentration:

X = The amount of plasma to be removed or added to obtain the desired concentration by volume or count.

A = Total volume of blood sample to be corrected in c.c.

B = Packed cell volume in c.c. per 100 c.c. of blood or the R.B.C. in millions.

C = Desired packed cell volume in c.c. per 100 c.c. of blood, or the R.B.C. in millions.

The following formula can be used when the cell volume or cell count is less than that desired. X = c.c. of plasma to be removed:

$$X = \frac{C \cdot B}{C} \times A$$

The following formula can be used when the cell volume or cell count is more than that desired. X = c.c. of plasma to be added:

$$X = \frac{B \cdot C}{C} \times A$$

Procedure.—1. Collect and adjust blood sample as described above. The interval between the collection of the blood and the starting of the test should be as short as possible, preferably not over one hour. If any clotting has occurred, the sample of blood is unsatisfactory for testing.

2. Carefully fill the sedimentation tube to the top graduation, avoiding bubbles. A description of some special tubes which are used for this test are listed below.

3. Immediately place the tube in a vertical position and note the time. It is not sufficient to place the tube in an ordinary test tube rack and depend upon the eye to determine its position. A special rack (see Fig. 83) should be used which will insure a vertical position of the tube and then it is necessary to place the rack on a level platform or table. The Westergren tube (Fig. 84) may be suspended after placing a cotter pin in the lumen at the top and closing the opening at the bottom with a rubber bulb or band (see Fig. 85).

FIG. 83.—SEDIMENTATION TUBE SUPPORT FOR WESTERGREN TUBES

4. Allow the tubes to stand at room temperature (22° to 27° C.) and observe the sedimentation of the erythrocytes by determining the distance in millimeters that the erythrocytes have settled from the top. Most of the tubes are graduated so that the reading can be made directly from the tube. If other tubes are used the measurement can be made with a millimeter rule.

Sedimentation Tubes.—*Cutler Tube.*—Length 70 mm.; internal diameter approximately 5 mm.; capacity 1 c.c.; graduated from 0 to 40 mm. in single milli-

meter divisions (see Fig. 75). The Cutler sedimentation pipet is shown in Fig. 87.

Westergren Tube.—Length 300 mm. graduated from 0 to 200 mm. in 1 mm. intervals; capacity approximately 1 c.c. (Fig. 84).

Wintrobe Tube.—Flat bottom glass tube 110 mm. long, with 3 mm. uniform bore, graduated from 0 to 10 cm. in 1 mm. intervals (see Fig. 88).

If a special tube is not available a 1 or 5 c.c. pipet can be used. The blood is drawn up to the desired height, plugging the lower end with rubber stopper and suspending with cotter pin. The amount of sedimentation is measured with a millimeter rule.

Sedimentation Rate (S.R.).—This represents the velocity of sedimentation per unit of time. It is common practice to express it in millimeters at the end of one hour.

Sedimentation Time (S.T.).—The time in minutes required for the erythrocytes to settle to the point of packing or until sedimentation has practically ceased. The normal sedimentation time is always a question of hours. It is seldom determined in routine work.

Fig. 84.—WESTERGREN BLOOD SEDIMENTATION TUBE.

Sedimentation Curves.—If readings are made at short intervals and plotted against time, curves will occur which can be divided into two phases, the first being that part of the curve representing the rapid and orderly sinking of the cells, and a second, a progressive slowing of the rate due to the concentration and packing of the cells in the bottom of the tube. For this reason the sedimentation rate should be determined during or at the end of first phase. Therefore, a tube with a column sufficiently high to permit the first phase to continue until the end of the time unit selected should be used. If, however, curves are desired, then a short tube such as recommended by Cutler should be used in which case the first phase will be ended within one hour in those cases showing marked increase in sedimentation rate. Cutler describes a method for plotting multiple readings against time resulting in certain curves to which he gives special significance (see Fig. 89).

Fig. 86.—CUTLER BLOOD SEDIMENTATION TUBE.
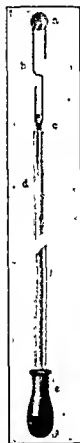
Fig. 85. — METHOD OF SUSPENDING THE WESTERGREN TUBE.

Normals.—The normal range for men is 0 to 8 and women 0 to 10. The normals are applicable to all tubes whereas abnormal readings in one tube are not necessarily comparable to readings in other tubes. When reporting the sedimentation rate the type of tube used should always be mentioned.

Factors Influencing the Sedimentation Rate.—(*a*) *Anticoagulants* with the probable exception of heparin influence the sedimentation rate. It is only when the anticoagulants recommended are used in proper doses is this influence eliminated to an acceptable degree.

FIG. 87.—BLOOD
SEDIMENTATION
PIPET

(*b*) The *bore of the tube* does not influence the rate with the exception that tubes less than 2 mm. in internal diameter are unsatisfactory because sedimentation is uneven in such tubes.

(*c*) The *length of the tube* is a factor in influencing the sedimentation rate. The longer the tube, the greater the rate; however, the distance traversed is not directly proportional to the distance to be traversed, *e.g.*, the sedimentation in a tube of 300 mm. may be only slightly greater than in a 100 mm. tube at the end of 1 hour. Greater changes in the sedimentation rate occur with shorter tubes due to the influence of packing.

(*d*) The *inclination of the tube* influences the rate. Deviation of the sedimentation tube from the perpendicular position causes an acceleration of the sedimentation rate.

FIG. 88.—WIN-
TROBE BLOOD
SEDIMENTATION
TUBE

(*e*) The *time interval between collecting the blood and starting the test* is a factor. A delay causes a decrease in the rate. Wintrobe and Andsberg found a significant difference after 4 hours using a 100 mm. tube. Boerner and Flippin report a significant difference at the end of 1 hour using a 200 m.m. tube. Bloods vary greatly in this respect; in some the difference in stability occurs much earlier than in others.

(*f*) The *temperature* at which the test is conducted influences the rate. The higher the temperature the greater the rate. Therefore it is important to conduct the test at about the same room temperature.

(*g*) The *concentration* of the cells is an important factor. The more dilute the blood the greater the rate. This factor can be eliminated by adjusting the concentration of cells by the methods described above.

(*h*) There is no doubt that other factors may influence the test.

METHOD FOR DETERMINING THE TONICITY (FRAGILITY) OF ERYTHROCYTES

Principles.—1. Normally human erythrocytes carefully collected without injury can remain for two hours at room temperature in solutions containing 0.42

BLOOD SEDIMENTATION TEST

Case No._____ Date_____19____

Name_____ Tube No._____

Address_____ Diagnosis_____

Check	GRAPH	CORRESPONDING CLINICAL CONDITION	SEDIMENTATION INDEX	SEDIMENTATION TIME
	Horizontal Line	Normal or Absolute Quiescence		
	Diagonal Line	Quiescence		
	Diagonal Curve	Slightly to Moderately Active	_____ MM.	_____ Min.
	Vertical Curve	Moderately to Markedly Active		

Sed. in MM	TIME IN MINUTES													Sed. Index
	0	5	10	15	20	25	30	35	40	45	50	55	60	

EXPLANATORY NOTES:
Sedimentation Index. Represents the total sedimentation of red blood cells at the end of 60 minutes expressed in millimeters. Normal value for men varies from 1 to 8 millimeters, with an average of 3 to 4; for normal women from 1 to 12 with an average of 5 to 6.
Sedimentation Time. Represents the time required for the "complete" settling of the red blood cells. Normal time always a question of hours. Of abnormal rates when reduced to minutes or less.
Horizontal Line. A straight line with a sedimentation index falling within normal limits. It shows sedimentation normal. The other graphs are always abnormal degrees.
Diagonal Line. A straight line with a sedimentation index beyond normal limits.
Diagonal Curve. A curve of gradual descent with a sedimentation index beyond normal limits and a sedimentation time of 35 to 50 minutes.
Vertical Curve. A curve of sharp descent with a sedimentation index beyond normal limits and a sedimentation time of 30 minutes or less.

FIG. 89.—BLOOD SEDIMENTATION CHART AND GRAPHS

Horizontal line represents clinically healthy individual; diagonal line represents clinically quiescent tuberculosis; diagonal curve represents clinically slightly active tuberculosis; vertical curve represents clinically marked active tuberculosis. (J. W. Cutler.)

to 0.44 per cent sodium chloride *before hemolysis begins,* while under these conditions *hemolysis is complete* in 0.36 to 0.32 per cent solutions.

2. The point of beginning hemolysis is called *minimal resistance,* while the point of complete hemolysis is called *maximal resistance.* The former is of more diagnostic value than the latter.

3. After splenectomy resistance increases; in various diseases of the blood, variations from the normal may occur.

Sanford's Method.—1. Place twelve small test tubes in a rack and mark them from 1 to 12.

2. To each tube add the amount of 0.5 per cent salt solution and water as indicated in Table VI. The 0.5 per cent salt solution is prepared by placing C. P. sodium chloride in an oven for thorough drying and then dissolving 0.5 gm. in exactly 100 c.c. of distilled water:

<div align="center">

TABLE VI

</div>

Tube No.	0.5% Saline	Water % NaCl
1.	1.25 c.c.	0 = 0.5
2.	1.20 c.c.	0.05 c.c. = 0.48
3.	1.15 c.c.	0.10 c.c. = 0.46
4.	1.10 c.c.	0.15 c.c. = 0.44
5.	1.05 c.c.	0.20 c.c. = 0.42
6.	1.00 c.c.	0.25 c.c. = 0.40
7.	0.95 c.c.	0 30 c.c. = 0.38
8.	0.90 c.c.	0.35 c.c. = 0.36
9.	0.85 c.c.	0.40 c.c. = 0 34
10.	0.80 c.c.	0.45 c.c. = 0.32
11.	0.75 c.c.	0.50 c.c. = 0 30
12.	0.70 c.c.	0.55 c.c. = 0.28

3. Take the tubes in a rack to the bedside of the patient. Obtain 1 or 1.5 c.c. of blood from a vein with a small dry syringe and No. 21 needle and at once add 1 drop to each tube (Fig. 90).

4. If some time must elapse before the blood can be added, it may be mixed with 5 volumes of a 1 per cent solution of sodium citrate in physiological saline solution. Mix well. Centrifuge. Discard the supernatant fluid. Add an equal volume of saline solution to the corpuscles (gives a 50 per cent suspension) and add 1 drop to each tube.

5. It is advisable to prepare a similar set of tubes, using the blood of a normal person as a control.

6. Allow the tubes to stand at room temperature for two hours.

7. Read the results noting the per cent of NaCl in which hemolysis begins and the first tube showing complete hemolysis.

8. Normal blood *begins* to hemolyze in 0.42 or 0.44 per cent sodium chloride and is *completely* hemolyzed in 0.36 to 0.32 per cent. When a control is used, a variation of 0.02 or 0.04 may be considered quite definite. Sanford found the average figures for beginning and complete hemolysis in 23 cases of hemolytic

jaundice to be 0.478 and 0.413 respectively; in chronic obstructive jaundice, 0.396 and 0.31. In secondary and pernicious anemia the figures vary only slightly from

FIG. 90.—FRAGILITY TEST; ADDING THE BLOOD

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

the normal, with a tendency to slight increase of resistance. In purpura, resistance is normal.

METHOD FOR DETERMINING THE PHAGOCYTIC POWER OF WHOLE BLOOD

Principle.—The ability of the blood to clear itself of bacterial invaders depends to a large extent upon the ability of the leukocytes to ingest and destroy them (phagocytosis). To measure this phagocytic power, bacteria are brought into contact with the living leukocytes of the blood and the ability of the cells to ingest the bacteria is determined.

Preparation of Bacterial Suspension.—Inoculate buffered broth with the organism to be tested, the amount depending upon the number of tests to be conducted. The broth is then incubated for 18 to 24 hours at the end of which time, if satisfactory growth is obtained, it is centrifuged. Remove the supernatant fluid and resuspend the sediment in 10 c.c. of sterile saline or buffered broth. Centrifuge a second time. Remove the supernatant fluid and resuspend again in 10 c.c. of sterile saline or buffered broth. Centrifuge a third time. Remove the supernatant fluid and re-suspend in 1 to 2 c.c. of saline or broth depending on the amount of sediment obtained. Make a bacterial count using the Petroff-Hausser counting chamber. Adjust the concentration of the bacterial suspension to a suitable value; for smooth pneumococci about 6 billion bacteria per c.c. and for staphylococci, about 800 million per c.c. have been found suitable. Other simpler methods for estimating the bacterial content may by experience prove sufficiently reliable. It is not necessary to take precautions against contamination during or after the preparation of the suspension, since it is used on the same day. Killed organisms can be used, but they often stain poorly and therefore make more difficult the examination of smears.

Anticoagulant.—Prepare a stock solution of *purified heparin* by dissolving 25 mg. in 5 c.c. of 0.85% salt solution. This solution can be sterilized by autoclaving. It has been found to keep well for a week or two without marked deterioration. The amount used with human blood is 0.05 c.c. of the stock solution which amounts to 0.25 mg. For dog blood, twice the amount, or 0.1 c.c. of the stock solution is used. Parallel tests of heparinized blood and blood without any anticoagulant resulted in the same degree of phagocytosis, thus showing that this anticoagulant did not interfere with phagocytosis. Although the purified heparin is recommended the less expensive impurified product can be used.

Procedure (Boerner and Mudd).[8]— Place 0.05 c.c. of stock solution of heparin in a test tube for each c.c. of blood to be obtained.

2. Obtain blood by venous puncture and place in tube containing the heparin. Mix gently.

3. Place 1 c.c. of heparinized blood in a short test tube, 15 mm. x 75 mm., and place in agitator bath (see Fig. 91). The water in the bath should be between 37° and 38° C.

4. Add 0.1 c.c. of bacterial suspension to the blood, note time and mix quickly by gentle agitation. Start agitator.

5. At regular intervals of time remove by means of a capillary pipet sufficient blood for making a smear in the same way as is usual for differential counts of blood. This can be done without stopping the agi-

FIG. 91.—BOERNER-MUDD SHAKING APPARATUS FOR PHAGOCYTOSIS

tator, by dipping the pipet in the blood and removing quickly. A new pipet should be used for each sample. The slides should be perfectly clean and the ones used for spreading the film should have the corners cut off, so that the width of the film will be somewhat smaller than the width of the slide. This is done so the edges of the film are more easily examined. The interval of time that has proven most satisfactory has been 3 minutes. In some instances, where phagocytosis is rapid, examinations must be made at one-minute intervals. The smears should be thin enough to dry rapidly.

6. Fix the smears in methyl alcohol for 10 minutes.

7. Stain the Giemsa stain. It is advisable to make a few extra slides at the end of the test for the purpose of testing the stain before staining the test slides.

8. When making smears by the method just described, it will be found that the neutrophils are much more numerous along the edge of the film. This

[8] *Am. J. M. Sc.*, 1935, 189:22.

arrangement of the cells facilitates the examination by making it easy to locate the cells, and prevents the possibility of counting the same cell twice. The smear is examined by focusing the edge at one end and examining the cells found along or near the edge, always moving the slide in one direction. If sufficient cells are not found then the other edge should be examined. The cells should be classified as positive or negative for phagocytosis. The results are expressed in terms of percentage of positive cells. At least 100 cells should be classified. Cells which are clumped so their cytoplasm cannot be clearly outlined or cells which are broken up or degenerated, should be omitted. Only cells which have organisms within their cytoplasm should be considered positive. When bacteria are merely adherent to the cell or appear partly ingested, the cell should be classed as negative.

9. A count which is more satisfactory when there is an abundance of phagocytosis is made by scoring the number of bacteria taken up by each of 100 leucocytes, a score of 3 being given to a cell containing 1 to 5 bacteria, 8 to a cell containing 6 to 10, 15 to a cell containing 11 to 20 and 30 to a cell containing more than 20 bacteria. The total score is added up and divided by 30; thus if each cell has ingested more than 20 bacteria the score will be 100.

10. Curves can be obtained by plotting the per cent of positive cells against the time intervals.

METHOD FOR EXAMINATION OF THE CELLULAR CONTENT OF BONE MARROW

Principle.—When disease of the hemopietic system is suspected and the blood picture is unusual or the changes insufficient to establish a diagnosis, the examination of the bone marrow is often of diagnostic value.

Procedure.—1. Anesthetize the skin locally over the sternum.

2. Incise the skin midline over the mid portion of the sternum down to the periosteum. The length of the incision should be about 1 cm.

3. Introduce through the incision a large bore needle with guard set at ¼ inch from point.

4. Push the needle through the anterior table of the sternum and aspirate a few drops of marrow into the needle only, not the syringe. (If the sternum is unusually dense a trephine may be used.)

5. Mix the aspirated marrow thoroughly with 0.3 c.c. of the patient's serum previously obtained.

6. Make smears on slides in same manner as for differential count.

7. Close the incision with stitch or adhesive and dress.

8. Figure 92 shows photomicrographs of bone marrow prepared by the method of Custer (see Chapter XXXVIII for technic and additional illustrations).

THE DAVIDSOHN PRESUMPTIVE TEST FOR INFECTIOUS MONONUCLEOSIS [9]

Principles.—The test is based on the agglutination of sheep erythrocytes by the heterophilic antibodies in the serum of patients with infectious mononucleosis.

[9] Am. J. Dis. Child., 1935, 49:1222 1231; Am. J. Clin. Path., 1935, 5:455.

aterials Required.—1. Test tubes: 75 mm. long and 9 to 10 mm. in the
le diameter.

Blood serum inactivated for 30 minutes at 56° C.

A 2% suspension of sheep red corpuscles. The preparation of the sheep
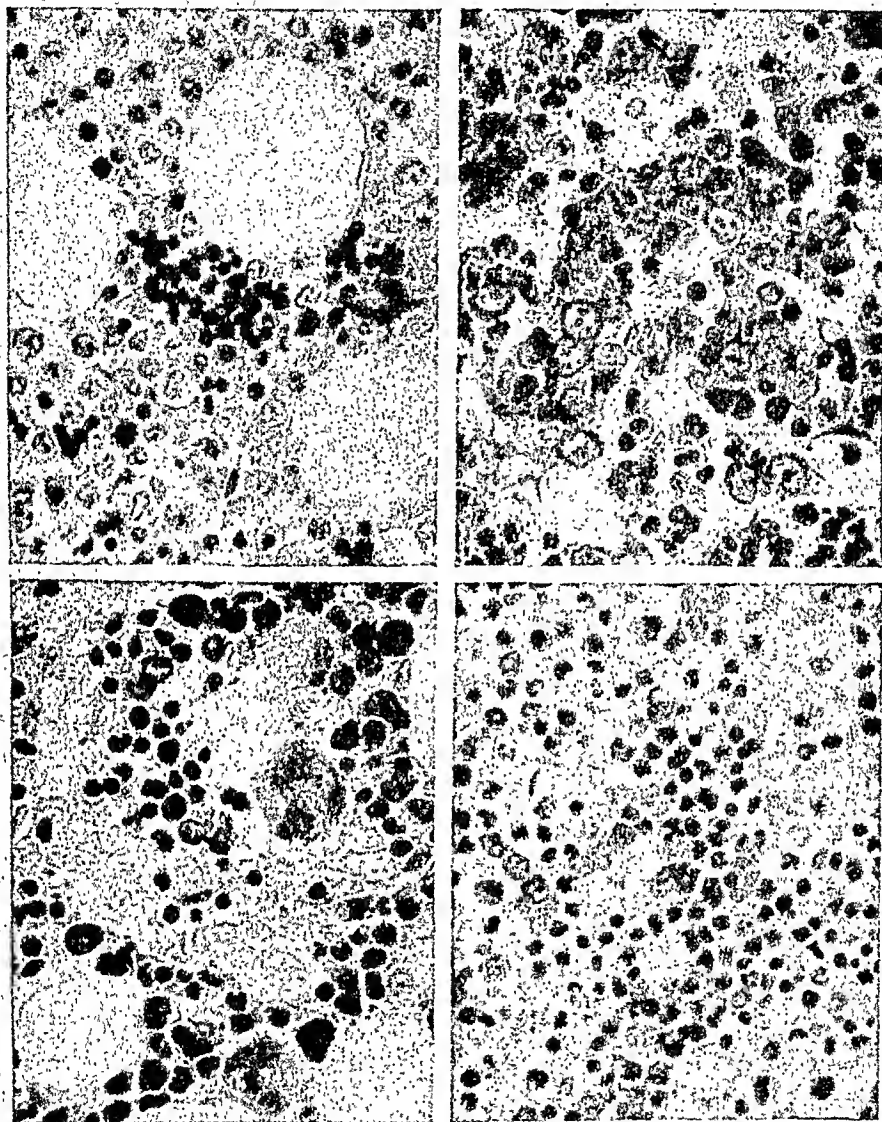
FIG. 92.—CELLULAR CONTENT OF BONE MARROW

Upper left: Normal sternal bone marrow; upper right: sternal bone marrow in pernicious
anemia; lower left: sternal bone marrow in hemorrhagic anemia; lower right: sternal bone
marrow in idiopathic hypochromic anemia.
(Courtesy of Dr. R. P. Custer)

corpuscle suspension is identical with the procedure outlined in the chapter on the
Kolmer Complement Fixation test for Syphilis. The sheep blood should be not
less than 24 hours old and not older than about one week, because later the cells

tend to become too easily agglutinable. The suspension must be prepared from cells that were washed on the same day.

Procedure.—The titration of the agglutinins is performed with 0.25 c.c. of the serum dilutions ranging from 1:7 to 1:7168. The best way to prepare these dilutions is to place 0.4 c.c. of physiologic sodium chloride solution in the first test tube and 0.25 c.c. in the remaining 10 test tubes. The final dilutions of serum are considered the titer.

1. To the first tube add 0.1 c.c. of serum.

2. Mix and transfer 0.25 c.c. to the second tube. Mix the second tube and transfer 0.25 c.c. to the third, etc., until the last tube is reached from which 0.25 c.c. are discarded after mixing.

3. Add 0.1 c.c. of a 2 per cent suspension of sheep red blood cells.

4. Shake the test tubes and leave at room temperature for two hours.

5. The results are read after shaking the test tubes. The shaking is continued until the entire sediment is suspended. The test tubes in which after shaking the cells remain in the form of a single clump are read as 3 plus. Those in which the cells break up into distinctly visible clumps and the fluid is clear and transparent, are called 2 plus. The reading of the 1 plus agglutination is best carried out by means of a low power objective of the microscope (f. i. a 32 mm. objective). The test tube is placed horizontally on the stage after the tube was shaken and in this way one can determine the end point of the agglutination with the greatest accuracy. However, after one acquires some experience, one can determine the end point fairly accurately with the naked eye. It is usually 1 to 2 dilutions lower than the titer determined with microscope. When time permits, it is advisable to repeat the reading after an overnight incubation in the ice-box. The titer is then usually 1 to 2 dilutions higher.

TABLE VII

TECHNIC OF TEST FOR INFECTIOUS MONONUCLEOSIS

Tubes	Saline c.c.	Serum c.c. Dilution	Serum Dilutions	2% Sheep Cells c.c.	Titer (Final Dilutions of Serum)	
1.	.4		1:5	.1	1:7	Shake tubes well; keep at room temperature for 2 hours and read
2.	.25	.25 of 1:5	1:10	.1	1:14	
3.	.25	.25 of 1:10	1:20	.1	1:28	
4.	.25	.25 of 1:20	1:40	.1	1:56	
5.	.25	.25 of 1:40	1:80	.1	1:112	
6.	.25	.25 of 1:80	1:160	.1	1:224	
7.	.25	.25 of 1:160	1:320	.1	1:448	
8.	.25	.25 of 1:320	1:640	.1	1:896	
9.	.25	.25 of 1:640	1:1280	.1	1:1792	
10.	.25	.25 of 1:1280	1:2560	.1	1:3584	
11.	.25	.25 of 1:2560 *	1:5120	.1	1:7168	
Control						
12.	.25			.1	—	

* Discard .25 c.c. from last tube.

6. One control is used consisting of 0.25 c.c. of physiologic sodium chloride solution and 0.1 c.c. of the sheep cell suspension.

The technic of the test is summarized in the table.

Interpretation of Results.—The finding of a titer of at least 1:224 in a person who did not receive an injection of horse serum or of horse immune serum in the recent past and who presents a clinical picture and hematologic findings suggestive of infectious mononucleosis indicates with a high degree of probability the presence of infectious mononucleosis.

A titer of over 1:224 should be considered as positive even if there is a history of horse serum administration. unless the patient is suffering at the time of examination from serum disease or unless he had recently gone through an attack of serum disease. Such titers were mainly encountered in the course of serum disease and for relatively short periods afterwards.

THE DAVIDSOHN DIFFERENTIAL TEST FOR INFECTIOUS MONONUCLEOSIS

Principles.—The heterophilic antibodies (antisheep agglutinin) in infectious mononucleosis are not of the Forssman type. They are not absorbed by a suspension of guinea-pig kidney. The heterophilic antibodies in serum disease are of the Forssman type and are readily absorbed by a suspension of guinea-pig kidney.

The antisheep agglutinins are absorbed by boiled beef red corpuscles from the sera of patients with infectious mononucleosis and with serum disease, but not from normal sera.

Preparation of Reagents.—1. *Guinea-Pig Kidney.*—The kidneys of the guinea-pig are kept frozen in the refrigerator until needed. They are then thawed and washed repeatedly in a physiological solution of sodium chloride until the washings are free of blood. They are now mashed into a fine pulp and used for absorption as a 20% suspension in physiological salt solution. The suspensions are boiled for one hour on the water bath and the loss by evaporation made up with distilled water.

2. *Beef Cells.* The beef red cells are washed 3 times. packed well in the centrifuge, suspended in four volumes of a physiological salt solution and boiled for one hour on the water bath. The loss by evaporation is made up with distilled water.

Enough phenol is added to the antigenic suspensions to make a 0.5 per cent solution. The antigens may be kept in the ice-box for many months without a noticeable change.

Absorption with Boiled Guinea-Pig Kidney Antigen.—1. Place in a test tube (85×13 mm.) 0.5 c.c. of the thoroughly shaken 20% suspension of boiled guinea-pig kidney.

2. Add 0.1 c.c of serum that has been heated for 30 minutes at 56° C.

3. Shake and let stand at room temperature for one hour, shaking at 15-minute intervals.

4. Centrifuge at 1500 revolutions for 10 minutes.

5. Remove the supernatant fluid with a capillary pipet.

6. To a row of six tubes (75×12 mm.) add 0.25 c.c. of physiological salt solution.

7. To the first tube add 0.25 c.c. of the absorbed serum.

8. Mix and transfer 0.25 c.c. to the second tube, etc. Discard 0.25 c.c. from the last tube. The serum dilutions are: 1:10, 1:20, 1:10, etc.

9. Add 0.1 c.c. of a 2 per cent suspension of sheep cells. Shake well. Final dilutions of serum are 1:14, 1:28, etc. Let stand at room temperature for 2 hours. Read.

Absorption with Boiled Beef Corpuscle Antigen.—Exactly the same procedure as above, using 0.5 c.c. of the thoroughly shaken 20% suspension. If it is necessary to begin with dilution of 1:7 as may be the case in serums with titers below 1:112, then add 0.2 c.c. of serum to 1 c.c. of the antigen suspension. For titration, omit the physiological salt solution from the first tube, but in the other tubes, place the usual amount of 0.25 c.c. From the absorbed serum, add 0.25 c.c. to the first and to the second tube. Proceed as above. Final dilutions are: 1:7, 1:14, etc.

Control with Unabsorbed Serum.—At the same time carry out a diagnostic test on unabsorbed serum according to the previously outlined technic to have a basis for comparison.

Interpretation of Results.—In the case of infectious mononucleosis, the absorption of the serum with the suspension of the guinea-pig kidney will effect a partial removal of the agglutinins for sheep red cells, but not less than one-fourth of the titer will remain: f. i. the titer before absorption 1:112, after the absorption with the guinea-pig kidney 1:28. If all or almost all of the agglutinins were removed then this speaks against infectious mononucleosis. The absorption with ox cells is a confirmatory procedure. The agglutinins for sheep red cells are completely or almost completely removed by beef cells.

Indications for the Differential Test.—1. A history of a recent injection of a horse immune serum or of serum disease in the recent past in a patient with a titer of heterophilic antibodies of 1:224 or over as determined with the presumptive test for infectious mononucleosis.

2. A borderline titer of heterophilic antibodies (1:56 or 1:112) as determined with the presumptive test for infectious mononucleosis.

METHODS FOR EXAMINATION FOR MALARIA

Malaria is a disease due to the invasion of the red corpuscles by animal parasites belonging to the genus Plasmodium.

Three species are of importance: *Plasmodium vivax,* producing tertian malaria, with paroxysms recurring every 48 hours; *Plasmodium malariae,* quartan malaria, with paroxysms recurring in 72 hours; and *Plasmodium falciparum,* producing malignant tertian or estivo-autumnal malaria, paroxysms recurring irregularly.

The life cycle of the parasites consists of a sexual phase, taking place in the body of a mosquito (anopheles), and an asexual phase within the red cells of man.

PLATE III

MALARIAL PARASITES

Wright's stain. × 1000 (1 mm. = 1 μ).
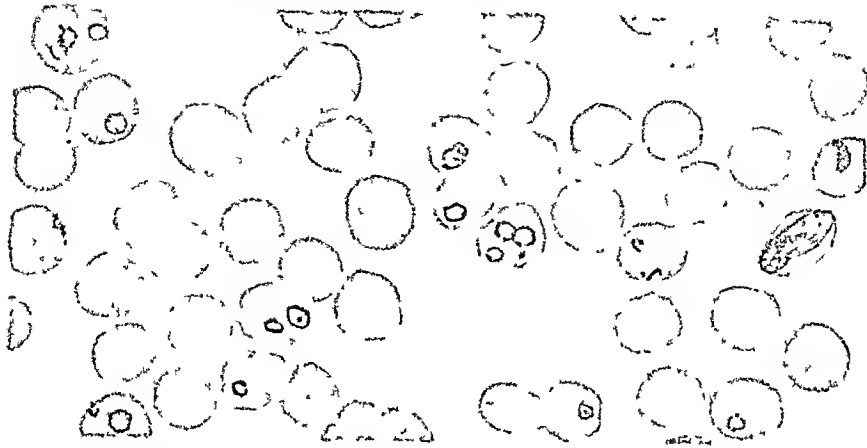
Fig. 1.—Estivo autumnal malaria; exact reproduction of a portion of a field showing an exceptionally large number of parasites.
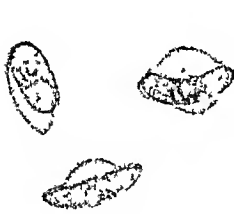
Fig. 2.—Estivo-autumnal game-tocytes.
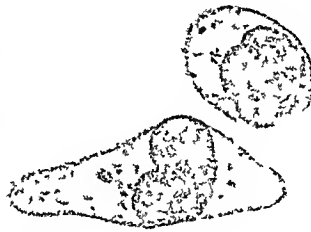
Fig. 3.—Leukocytes with engulfed pigment.

Fig. 4.—Quartan parasites.

A   B   C
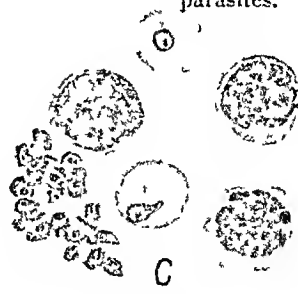
Tertian parasites. A, Eight hours after chill, showing malarial stippling, five young parasites, and one gametocyte, from two slides; B, twenty-four hours after chill, five half-grown parasites: one gametocyte; C, during chill, one presegmenter. two segmenters, a cluster of freshly liberated merozoites, and two very young parasites, from one slide

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co. Philadelphia.)

The laboratory diagnosis is established by (1) Direct examination of fresh blood. (2) Examination of stained blood smears (*a*) thin smear method (recommended); (*b*) thick smear method; (*c*) concentration method. (3) Culture (usually omitted in routine examination).

Fresh Wet Blood Method.—1. Use perfectly clean and grease-free cover glasses or slides.

2. Puncture the finger or lobe of the ear and take up a small drop of blood by touching same with center of a cover glass.

3. Place on slide so that the blood will spread out in a thin film.

4. If examination is prolonged, seal edges of cover glass with melted vaselin to prevent drying.

5. Examine at once with oil-immersion objective.

6. The best time for examination is six to eight hours after a paroxysm, but the parasites can be found at other times and the examination should be made without any special reference to the occurrence of paroxysms.

Thin Smear Method.—1. Smears are prepared as for differential leukocyte count but so thin that the red cells lie flat and well separated.

2. Fix and stain with Wright's or Giemsa's stain in same manner as staining for differential leukocyte counts.

3. Dry and examine with 1/12 oil-immersion objective.

4. The smears must be well stained for satisfactory results. Unless the nuclei of leukocytes are well stained and have the proper reddish-purple tint due to proper staining of the chromatin, the chromatin of the plasmodia will not be properly stained. Good and poor areas may occur on the same slide.

5. Malaria plasmodia are in the erythrocytes, and no object should be considered as a probable plasmodium unless it is so situated.

6. With Wright's or Giemsa's stain the chromatin of the parasite will take on a ruby red color, the protoplasm of the organism a sky-blue (pale blue), the pigment a black or dark brown, and the blood platelets and the nuclei of the leukocytes a reddish purple.

Great care should be exercised to avoid mistaking the blood platelets accidentally superimposed upon red cells for malarial parasites. These platelets are frequently surrounded by an unstained halo. Precipitated stain, dirt, bacteria, etc., may constitute other sources of error.

Precipitated stain granules may be removed by immersing the slide for a second or two in 95% alcohol and immediately washing with distilled water.

Thick Smear Method of Barber and Komp.—1. It is essential to carefully clean the skin with alcohol and gauze in order that the blood be free of dirt, bacteria, dust or other débris. The slides should be perfectly clean.

2. Put on a drop three or four times as large as used for ordinary thin blood smears. Spread by dragging the drop on the surface of the slide with the sticking needle or corner of another slide.

3. The smears should be dried enough to make them adhere, but too much drying will prevent a clear staining of the parasites. In ordinary summer weather

shaped, of a blue gray color, with considerable chromatin, and pigment near the center arranged more diffusely.

In estivo-autumnal malaria smears of the peripheral blood will show only *the rings* and *the crescents*.

METHODS OF EXAMINATION FOR MICROFILARIA

The filaria infest the deeper tissues of man and require an insect intermediate host.

The following species must be differentiated: (1) Filaria bancrofti (*Wunchereria bancrofti, Filaria sanguinis hominis*); (2) *Loa loa* (*Filaria loa, Filaria oculi*); (3) *Filaria perstans* (*Acanthocheilonema perstans*).

The diagnosis is established by examining the peripheral blood for the microfilaria as follows: Examination of fresh unstained blood (a) by direct method (b) by concentration method (c) by staining method.

Direct Method.—1. Puncture finger or lobe of ear and place large drop of blood on slide.

2. Immediately cover with coverglass and examine with low-power lens.

3. The larvae can be located by the disturbance they produce among the corpuscles.

Concentration Method.—1. Collect 1 c.c. of blood from ear or finger puncture in 5 c.c. of 2 per cent acetic acid.

2. Mix well and centrifuge.

3. Spread sediment on slide, cover with coverglass and examine with low-power lens.

Staining Method.—1. Make blood smears in usual manner or from sediment obtained by the above concentration method.

2. Dry, fix and stain by one of the methods for staining blood smears.

Aids for the Species Identification of the Microfilaria.—The microfilaria are easily detected in the peripheral blood due to their large size, and their intentionless lashing movement, which agitates the red cells and immediately attracts the eye when specimens are examined under the low power of the microscope.

*Microfilaria Bancrofti.—*The *M. bancrofti* is a sheathed embryo. It measures 300 by 7.5 micra. It forms graceful curves, is regular in outline, and is rarely angleated. The head is the same width as the body. The body matrix, which is made up of numerous nuclei, does not extend to the extreme limits of the tail, which is pointed. The embryos have a nocturnal periodicity. The blood should be examined from between 9 and 12 P.M. (See Figure 93.)

*Microfilaria Loa.—*M. Loa measures 250 by 7 micra. It is a sheathed embryo, the body matrix extending to the extreme limits of the tail, and ends squarely at the head. Its outline is slightly irregular, the head slightly flattened and broader than the body. Its curves are not graceful. It has a diurnal periodicity. The blood should be examined at mid-day.

*Microfilaria Perstans.—*This embryo is unsheathed, that is, the body cavity containing the nucleus is not covered by a surrounding layer or sheath. The head is

blunt, the organism measures 200 by 5 micra. This embryo does not exhibit periodicity, and is usually found in the peripheral blood at all times.
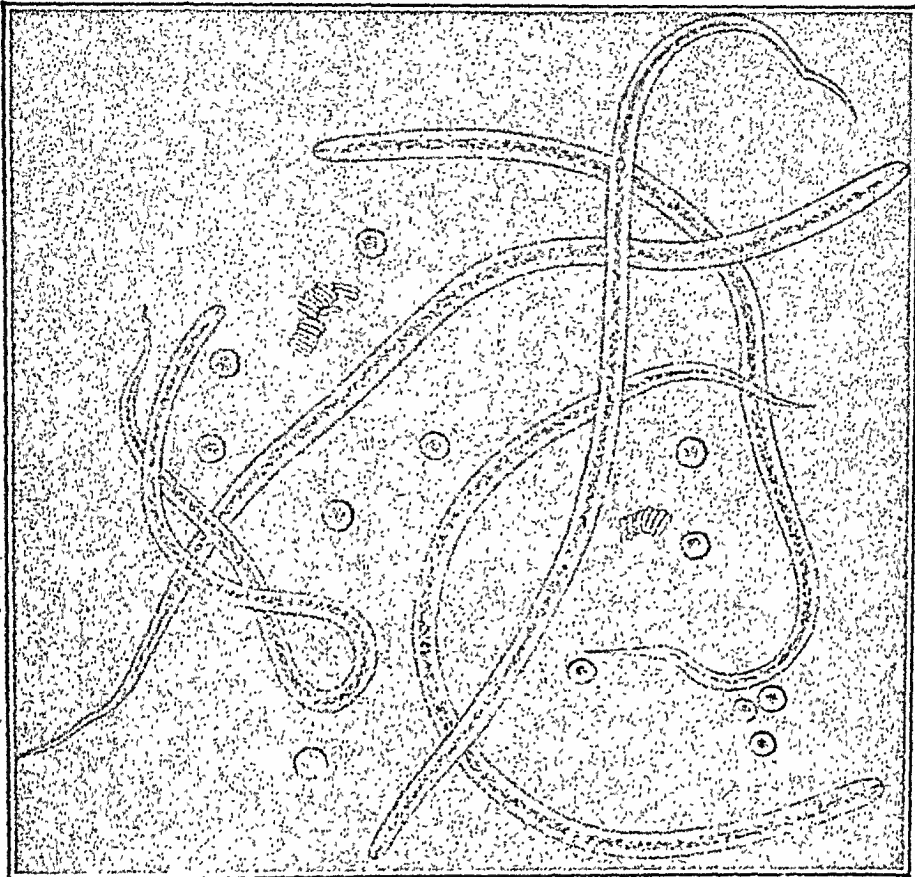
FIG. 93.—LARVAE OF FILARIA BANCROFTI

(After Railliet. From Braun, *Die thierischen Parasiten des Menschen*, Bale Sons and Danielsson, London.)

METHODS OF EXAMINATION FOR TRICHINELLA SPIRALIS

Examination of Blood for Larvae.—1. The larvae are sometimes found in the blood between the sixth and twenty-second days after the onset of symptoms.

2. A high eosinophilia is usually seen in differential leukocyte counts.

3. Obtain 10 c.c. of blood by venous puncture.

4. Mix thoroughly with 25 c.c. of 2 per cent acetic acid.

5. Centrifuge thoroughly.

6. Examine the sediment either moist by placing a drop on a slide and covering with cover glass or by smears stained with Wright's stain.

7. The larvae are easily recognized.

8. The results will be positive in about 50 per cent of the cases.

9. The larvae are also sometimes encountered in the spinal fluid after thorough centrifuging and preparing smears of sediment.

METHODS OF EXAMINATION FOR TRYPANOSOMES

Several important diseases of man and the lower animals are caused by species of the genus Trypanosoma:

TABLE VIII

Disease	Host	Vector	Trypanosome
Sleeping sickness (African)...	Man	Tsetse fly (G. palpalis)	T. gambiense
Sleeping sickness (Rhodesian)..	Man	Tsetse fly (G. morsitans)	T. rhodesiense
Chaga's disease	Man	Kissing bug (Triatoma megista)	T. cruzi
Surra	Horse, mule, camel	Horse fly	T. evansi
Nagana	Domestic animals	Tsetse fly (G. morsitans)	T. brucei
Souma (African)	Domestic animals	Tsetse fly (G. palpalis)	T. vivax
Dourine	Horse	Direct by coitus	T. equiperdum

The laboratory diagnosis is established by: (1) examination of the blood for the adult parasites: (a) stained blood smears and (b) concentration method. (2) Examination of material from lymph nodes for the parasites. (3) Examination of the spinal fluid. (4) Animal inoculation. (5) Complement fixation test.
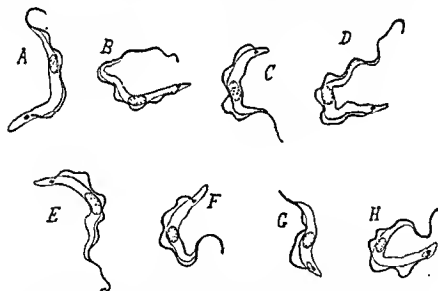
FIG. 94.—THE MOST IMPORTANT TRYPANOSOMES PARASITIC IN VERTEBRATES

A. Tr. lewisi. B. Tr. evansi (India). C. Tr. evansi (Mauritius). D. Tr. brucei. E. Tr. equiperdum. F. Tr. equinum. G. Tr. dimophon. H. Tr. gambiensi. × 1500. (After MacNeal.)

Stained Blood Smears.—1. Prepare blood smears same as for differential blood count.

2. Stain with Wright's stain.

3. The organisms are never found within the cells but are always free in the blood plasma (Fig. 94).

4. In the early stage of sleeping sickness and during the febrile stage of Chaga's disease, the organisms can usually be found by this method.

Concentration of Blood.—1. Thoroughly centrifuge 10 c.c. of citrated blood.

2. Make smears from the leukocytic cream layer, which is just above the packed red cells.

3. Stain with Wright's stain.

Examination of Lymph Glands.—1. Aspirate a lymph gland with a large hypodermic needle and syringe. It is sometimes necessary to first inject a few drops of sterile saline solution.

2. Prepare smears and stain with Wright's stain.

3. This method is recommended when the peripheral blood examination is negative for *Trypanosoma gambiense*.

Examination of Spinal Fluid.—1. Make spinal puncture and withdraw about 10 c.c. of fluid.

2. Centrifuge for fifteen minutes.

3. Prepare smears of the sediment and stain with Wright's stain.

4. The spinal fluid examination is useful in the late stages of sleeping sickness when the other methods are negative.

Animal Inoculation.—1. Inoculate white rats intraperitoneally with 1 c.c. of blood or tissue juice from suspected case. Rats and mice are particularly susceptible. Guinea-pigs and rabbits are also, but to a less extent. For *Trypanosoma gambiense* monkeys are preferred.

2. Make daily blood smears. In positive cases the trypanosomes will appear between the third and fourteenth day and remain in the blood stream quite constantly.

Complement-Fixation Test.—This test has been found useful in the diagnosis of dourine and the detection of carriers among the lower animals. Horses may tolerate an infection for one to three years, during which time they are capable of conveying the disease and yet remain normal in health and general appearance, and this method of diagnosis is, therefore, invaluable (Tyzzer). The test is similar to any other complement-fixation test except for the preparation of the antigen, a description of which is given in the section on serology. The test gives a group reaction. so the same antigen can be used for all species.

The Species Identification of the Trypanosomes of Man.—The Trypanosomes are the most highly developed of the hemoflagellates. For species identification the worker must be familiar with the structural details described in the paragraph below:

The parasites have a fusiform body with pointed ends. They are flattened from side to side. In the central part is a large nucleus. In the posterior end a small chromatic mass is found called the "parabasal body." Immediately adjacent is a blepharoplast, from which an undulating membrane and marginal flagellum arise. The flagellum in most species, after forming the edge of the undulating membrane, extends beyond the anterior end as a free process of varying length. In Figure 94 these structures have been represented diagramatically.

This test is not specific, the reaction being positive in tuberculosis, leprosy and malaria.

Aids to the Identification of Leishmania.—The diagnosis of Leishmaniasis usually depends upon finding the so-called "Leishman-Donovan" bodies (Leishmania forms). These are 2 to 4 micra in diameter, are usually oval or rounded in shape, and are found within the large reticulo-endothelial cells of the tissue or monocytes of the blood. They are said to somewhat resemble a cockle shell, because of the large eccentrically placed chromatin bodies. The larger or nucleus is round or oval, in front of which is a rod-shaped deep-staining body, the parabasal body and basal granule.

Stained with Leishman's or Wright's stain, the protoplasm is a pale blue, the nucleus stains dark, the parabasal body still darker.

In culture, the organisms develop heptomonad forms 14 to 20 micra in length. Some are fusiform in shape, with a centrally-placed oval nucleus and parabasal body at the anterior end, from which extend anteriorly the varying-length flagellum. No undulating membrane is present.

CHAPTER V

METHODS FOR THE EXAMINATION OF URINE

COLLECTION AND PRESERVATION

1. If any dependable data are desired regarding the quantitative composition of the urine, the examination of the mixed excretion for twenty-four hours is generally necessary. In collecting the urine the bladder may be emptied at any given hour, e.g., 8 A.M., the urine discarded and all the urine voided from that hour up to and including that passed the next day at 8 A.M. saved, thoroughly mixed and sample taken for analysis.

2. Powdered thymol is not a wholly satisfactory preservative, evidence having been presented to show that it interferes with the quantitative estimation of sugar, acetone, and diacetic acid. It also may give a confusing reaction in the nitric acid test for albumin.

3. Toluol overlayed on the urine is a very satisfactory preservative.

4. In certain pathological conditions, it is desirable to collect both day and night specimens. Urine voided between 8 A.M. and 8 P.M. may be taken as the day sample and that voided between 8 P.M. and 8 A.M. as the night sample.

5. The qualitative testing of urine samples collected at random, except in a few specific instances, is of no particular value in so far as giving accurate knowledge of the exact urinary characteristics. In the great majority of cases, a sample of the mixed twenty-four-hour specimen will give the most helpful data.

6. Single specimens continue to be mostly employed because of greater convenience in collection but should be used mainly for routine qualitative tests.

7. Single specimens collected at varying times in the day may yield different results, especially in amounts of sugar and albumin; this is a frequent reason for varying reports from different laboratories examining specimens of urine of the same person collected at different times. *Single specimens should be labeled with the time of voiding.* Specimens voided two or three hours after a meal are likely to contain most sugar or albumin; those passed first in the morning are least likely to contain them.

8. Different methods and variations in technic and skill, however, probably account for most of the discrepancies in reports from different laboratories.

9. Containers used for collection of urine should be *chemically clean* and preferably sterile. Careful cleaning is especially required in hospital laboratories to avoid the possibility of carrying over traces of albumin and sugar. Traces of syrup in insufficiently washed medicine bottles are sometimes responsible for mistakes.

10. Contamination with vaginal discharges may account for the presence of

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albumin and pus; contamination with menstrual discharges may account for presence of albumin and blood. Both should be carefully avoided, as well contamination with feces.

11. From 3 to 8 ounces of urine should be submitted for ordinary examinat

12. Urine to be examined for tubercle bacilli may be voided, although the are chances of contamination with smegma bacilli. Urine for other bacteriolog examinations should be collected aseptically by sterile catheter into sterile tainers (*without a preservative*), as it is almost impossible otherwise to avoid

Fig. 97.—Boerner's Urine Rack

terial contamination, especi with *B. coli* and staphylococc

Methods.—1. Give specifi rections for collecting the four-hour specimens. A large tle or other container kept i cool place may be emplo The total amount should be c *fully mixed and measured* an to 8 ounces submitted for ex ination. A preservative may required.

2. In hospital laboratories urine rack devised by Boerne recommended (Fig. 97).

3. Special methods must be employed for the collection of urine from infa

Preservatives.—Decomposition sets in rapidly, especially in warm weather, greatly interferes with all examinations. An ideal preservative should prevent growth of bacteria and molds; should not interfere with the accuracy of physi chemical and microscopical examinations; should be readily soluble, of low and preferably a solid. For *quantitative* tests the total twenty-four-hour urin advisable and, unless kept at a low temperature, a preservative is gener required:

1. *Thymol*, if used, should not exceed 0.1 gram per 100 c.c. of urine. An ex may interfere with albumin determinations. It is not as good as formerly mised and is unsatisfactory when urine contains sugar, acetone or diacetic a also when urine is to be examined for phenol and quantitatively for phosph or magnesium.

2. *Formalin in proportion of 2 to 4 drops to the ounce is the most satisfac of all, especially for the preservation of the formed elements.* An excess interfere with tests for indican, albumin and sugar, and produce a precipitate

3. *Boric acid*, 5 grains for each 4 ounces, delays decomposition but may inter with sugar determination and precipitate rhombic crystals of uric acid.

4. *Toluol* may be used, especially for specimens to be examined for ace and diacetic acid. Simply add enough to form a thin layer on the surface. It very satisfactory preservative **for routine use.**

5. Chloroform is the least satisfactory and should not be used as it interferes in sugar determination and microscopical examinations.

PHYSICAL EXAMINATION

Amount.—1. The normal volume of urine passed in twenty-four hours averages about 1200 to 1500 c.c.; women normally excrete less than men. More urine voided during the day than during the night.

2. *Polyuria*—increase in the amount voided.

Oliguria—decrease in the amount voided.

Anuria—greatly decreased or suppressed.

Nocturnal polyuria—increase in the amount of urine passed during the night.

Color.—1. The color of urine is subject to wide variations but possesses some diagnostic importance.

2. Normally it is yellow or reddish-yellow (amber), due to the presence of several pigments, chiefly urochrome.

3. Color depends largely upon the concentration of urine. Dilute urines are usually pale while concentrated urines are dark. Acid urine is usually darker than is alkaline urine.

4. Color may be greatly changed by abnormal pigments and by various drugs and poisons as follows:

Blood: red or brown; smoky
Bile: yellowish or brown, turning greenish; yellow foam
Chyle: milky
Methylene blue: greenish-blue
Phenols: olive-green to brownish-black, etc.

5. For uniformity in recording color, Vogel's scale is recommended, the urine being filtered and viewed by transmitted light in a glass 3 or 4 inches in diameter: pale yellow, light yellow, yellow, reddish-yellow, yellowish-red, red, brownish-red, reddish-brown and brownish-black. To these may be added greenish-yellow, olive, milky, etc.

Odor.—1. Normal urine has a characteristic odor which is more marked in concentrated urines.

2. Abnormal odors:

Ammoniacal: due to decomposition.

Fruity or *sweetish*: noted in diabetes, probably due to acetone.

Fecal: contamination with feces or *B. coli*. Various *foods* and *drugs* impart peculiar odors. Asparagus produces an offensive odor due to the presence in the urine of methyl mercaptan.

Transparency and Sediments.—1. Freshly passed urine is usually clear or transparent, but may be cloudy, due to the presence of phosphates or pus. The former disappears upon the addition of acid; the latter does not but may become gelatinous (Donné's test). A freshly passed urine may also be cloudy with bac-

teria or comparatively clear with numerous shreds of mucopurulent material (chronic urethritis).

2. A record of the transparency is only of value in comparatively fresh specimens. All become cloudy with bacteria and alkaline salts upon standing as the result of decomposition.

3. Upon cooling and standing all specimens develop a faint cloud of mucus, leukocytes and epithelial cells which settle to the bottom—the so-called "nubecula." This has no significance.

4. Acid urines may develop a white or pinkish sediment of amorphous urates.

5. Alkaline urines may develop a heavy white sediment of amorphous phosphates.

6. Pus gives a heavy mucoid whitish sediment.

7. Blood gives a reddish-brown smoky sediment.

8. Bacteria give a uniform cloudiness which cannot be removed by ordinary paper filtration.

9. The following terminology is recommended:

(a) Clear, slightly cloudy, cloudy, very cloudy.

(b) Sediment: Slight, moderate or heavy; white, pinkish, red, brown, reddish-brown, etc.; shreds present or absent.

DETERMINATION OF REACTION

1. Normally freshly voided urine is acid in reaction, the pH ranging from 4.8 to 7.5 with a general average of 6. Twenty-four-hour specimens are less acid than freshly passed urine and may be neutral or even slightly alkaline as a result of standing.

2. Freshly passed urine may be neutral or alkaline as the result of the administration of alkalis, retention with "ammoniacal decomposition," etc.

3. Diet influences the reaction.

4. The urinary acidity may be decreased or the urine may become alkaline for some time after a meal, due to the withdrawal of hydrogen ions from the blood during the secretion of free HCl by the stomach. The occurrence of this so called "alkaline tide" may be employed as an indirect indication of the occurrence or nonoccurrence of gastric secretion of free HCl.

Litmus Test.—For ordinary purposes the reaction may be determined with good grades of blue and red litmus papers (Squibb's recommended):

Blue turning red: acid
Red turning blue: alkaline
No change in either: neutral
Changes both red and blue: amphoteric

Titratable Acidity (Folin-Wu).—1. Use a sample of mixed twenty-four hour urine as fresh as possible and accurately measured.

2. Place 25 c.c. in a small flask or evaporating dish. Add 2 drops of 0.5 per cent alcoholic solution of phenolphthalein and 15 grams of neutral finely pulverized potassium oxalate.

3. Shake vigorously for two minutes.

4. Immediately titrate with N/10 sodium hydroxide solution, shaking after each addition, until the first permanent pink color appears.

5. Read off amount of N/10 sodium hydroxide used.

6. Multiply by 4 to estimate amount required for 100 c.c. of urine and report accordingly (normally 25 to 40 c.c.).

7. Calculate and report amount required for total twenty-four-hour specimen. Normally 300-600 c.c. (may be less; depends largely on diet).

DETERMINATION OF HYDROGEN ION CONCENTRATION

True acidity or hydrogen ion concentration is preferred and may be determined with the indicator solutions of Clark and Lubs. Fresh clear urine should be used and the technic is that described in Chapter XVII for determining the pH of culture média. The normal values lie between 4.8 and 7.5, with an average of about 6.0 (for vegetarians about 6.6).

DETERMINATION OF SPECIFIC GRAVITY

1. The normal average is from 1.015 to 1.020. Pathologically it may vary from 1.001 to 1.060. If the specimen contains but a small or average amount of sediment it makes but little or no difference whether the urine is mixed up or the specific gravity taken without mixing in order to use the sediment later for microscopical examination. If, however, there is a large amount of sediment the specific gravity is almost always increased by about 0.002 after thorough mixing.

2. For ordinary determinations the Squibb urinometer (Fig. 98) may be used but the urinometer used with the immiscible balance is probably the best on the market. It settles down quickly after spinning without bobbing or swaying, and its special scale makes it much easier to read. With the Squibb urinometer the technic is as follows:
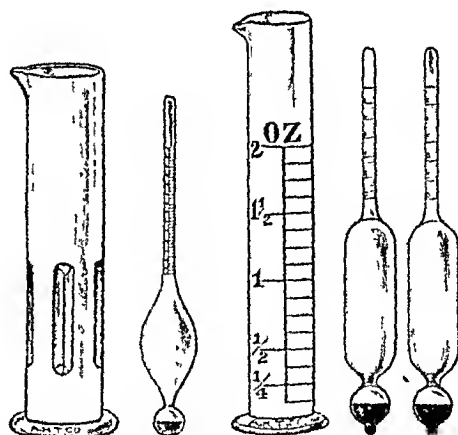
FIG. 98.—SQUIBB URINOMETER   FIG. 99.—VOGEL URINOMETER

(a) Fill the cylinder without producing bubbles. The specific gravity may be taken without having first mixed the urine.

(b) Float the urinometer so that it does not *touch the bottom or sides*.

(c) Make the reading from the bottom of the meniscus.

(d) The instrument is adjusted for readings at 22.5° C. For accuracy add 0.001 to the reading for each 3° C. above this temperature and subtract 0.001 for each 3° C. below, although moderate reduction in temperature does not influence the specific gravity as much as increased temperature.

3. The Vogel urinometer (Fig. 99) is more accurate and consists of two spindles, graduated respectively from 1.000 to 1.025 and from 1.025 to 1.050.

4. For small amounts of urine, dilute with an equal volume of distilled water, mix and take specific gravity. Multiply the last two figures by 2. By this method he specific gravity is usually 0.001 to 0.002 higher. The Saxe urino-pyknometer Eimer and Amend) may be used if at least 3 c.c. of urine are available.

5. In the case of urine containing large quantities of protein, correction should
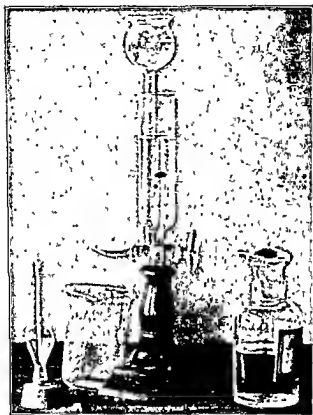
FIG. 100—EXTON'S IMMISCIBLE BALANCE

A drop of blood is shown in suspension. A special hydrometer is shown at the left.

be made for the latter by subtracting, 0.003 times grams of protein per 100 c.c., from the observed specific gravity.

6. The Exton immiscible balance (Fig. 100) supplied by the Emil Greiner Company may be employed for determining the specific gravity of drops of urine when only very small amounts are available, as in ureteral catheterization. The method and instrument are based on the principle of suspending the urine in an immiscible medium of the same specific gravity which is then determined by the usual methods, making possible rapid manipulations with minimal loss of material.

The cylindrical mixing chamber is partly filled with a mixture of varuolene

(petroleum ether) and carbon tetrachloride with a specific gravity of about 1.012. The side thistle tube is filled with varuolene and the carbon tetrachloride is kept at hand in a drop bottle. A drop of the urine is immersed in the mixture, which is then easily and rapidly varied by means of the stopcock and dropper, so that the urine remains suspended near the middle. The specific gravity of the mixture is then taken by means of the Exton hydrometer, which gives the specific gravity of the urine.

ESTIMATION OF TOTAL SOLIDS

1. If the total output of urine for twenty-four hours is reported in ounces, multiply the last two figures of the specific gravity by the number of ounces voided and to the product add one-tenth of itself. This gives the total solids in grains. Example:

> Twenty-four-hour output = 37 ounces
> Specific gravity (at 25° C.) = 1.014
> $14 \times 37 = 518 + 51.8 = 569.8$ grains

2. If the twenty-four-hour specimen is reported in c.c. multiply the last two figures of the specific gravity by Long's coefficient, 2.66; then multiply by the total output and divide by 1000, which gives the total solids in grams. Example:

> Twenty-four-hour output = 1120 c.c.
> Specific gravity (at 25° C.) = 1.018
> $2.66 \times 18 = 46.8$ gm. in 1000 c.c. of urine
> $\dfrac{46.8 \times 1120}{1000} = 52.4$ gm. in 1120 c.c.

3. The normal output for an adult of 150 pounds is about 60 grams or 950 grains. The above methods are only approximately correct but suffice for clinical purposes.

4. The output of urinary solids is influenced by body weight, diet, exercise, age, metabolism and kidney function.

QUALITATIVE DETECTION OF ALBUMIN

Principles.—Normal urine contains a trace of albumin which is too slight to be detected by the simple tests in general use, a large number of which have been described. All depend upon its precipitation by chemical agents or coagulation by heat. All precipitate both serum albumin and serum globulin and do not differentiate between these two proteins. Most are subject to some error largely due to the precipitation of mucin or other constituents. All require the use of clear specimens, preceded by filtration if necessary, in order to detect small amounts of albumin. The methods here given are recommended for ordinary routine work.

Methods for Filtering.—As a general rule simple filtration through ordinary filter paper is sufficient unless cloudiness is due to bacteria. Very large numbers

of bacteria and especially dissolved organisms in alkaline urine may yield faint traces of albumin. They are difficult to remove but this may be accomplished sufficiently for testing by centrifuging or by adding about one teaspoonful of purified talc, infusorial earth or animal charcoal to each 2 or 3 ounces, shaking well and filtering through two thicknesses of filter paper. Some albumin is also removed by adsorption.

Methods for Recording Reactions.—A wide diversity of methods for reporting qualitative tests are in use; they account in large part for discrepancies in reports from different laboratories. A uniform method and terminology are urgently needed. The following are recommended:

> — = negative.
>
> ± = very slight trace. Cloudiness or ring can just be seen against a black background.
>
> + (1) = slight trace. Cloud is distinct but not granular; no definite flocculation. Or the ring is sufficiently definite to be seen without a black background.
>
> + + (2) = moderate trace. Cloud is distinct and granular without definite flocculation. Or the ring is dense but not wholly opaque when viewed from above. Represents about 0.1 per cent of albumin.
>
> + + + (3) = heavy cloud. Cloud is dense with marked flocculation or the ring is heavy, wholly opaque and sometimes curdy. Represents about 0.2 to 0.3 per cent.
>
> + + + + (4) = very heavy cloud. Heavy precipitate to boiling solid; or very dense ring. Represents 0.5 or higher per cent of albumin; 3 per cent albumin boils solid.

Sulphosalicylic Acid Test.—1. Place 1 c.c. of urine in a test tube. If urine is not clear it should be filtered.

2. Add 1 c.c. of the reagent.

REAGENT

Sulphosalicylic acid .... 30 gm.
Water to make .... 1000 c.c.

3. Allow to stand 10 minutes.

4. If cloudiness does not develop, albumin is absent and the reaction is negative. Any cloudiness indicates the presence of albumin, the density depending upon the amount present. The reaction may then be recorded by the symbols described above.

If the urine is cloudy and cannot be cleared, the reaction should be compared with a tube containing water and urine. A distinct difference will be noted if albumin is present. This test is quite sensitive and highly recommended for routine work.

Purdy's Test.—1. Fill a thin-walled test tube half full with urine.

2. Add about one-sixth its volume of saturated water solution of sodium chloride and 5 to 10 drops of 50 per cent acetic acid.

3. Mix well and boil the upper portion over a Bunsen burner (Fig. 101). A holder is unnecessary. Rotate or shake gently by heating to prevent cracking of the tube by condensation of steam.

4. A cloud (best seen against a dark background) denotes the presence of
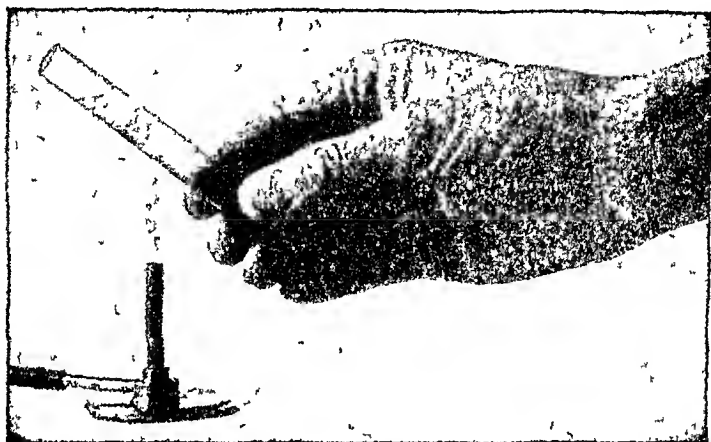
FIG. 101.—BOILING URINE

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

albumin (Fig. 102) and the results are recorded as described on page 134, or compared with standards described below.

5. This is a valuable routine test, as the addition of the sodium chloride raises the specific gravity and prevents precipitation of mucin. Bence-Jones protein may produce a cloud which disappears upon cooling.

6. This method may be adapted to the testing of a large number of samples at one time by using numbered tubes and placing in each about 5 c.c. of urine. 1 c.c. of sodium chloride solution and 5 drops of acetic acid, followed by mixing and placing in a boiling water bath for five minutes with the water above the level of the contents of the tubes.

Heat and Acid Test.—1. Boil about 5 c.c. of filtered urine in a test tube for one or two minutes. Hold with a clamp or a piece of filter paper folded around the neck.

2. Add 1 or 2 drops (no more) of concentrated nitric acid or 3 to 5 drops of dilute acetic acid.

3. A white cloud now disappearing is due to earthly phosphates. Effervescence is generally due to carbonates from the food.

4. A very faint trace of albumin may appear only upon the addition of the acid. Larger traces appear upon boiling and may become heavier upon the addi-

tion of the acid. The addition of too much acid may dissolve faint traces of albumin and give a falsely negative reaction.

Fig. 102.—Cloud of Albumin Seen Against a Dark Background

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

5. For the routine testing of a large number of samples by this method, use numbered test tubes and place in a boiling water bath for at least five minutes. Add the acid to each, mix gently and record the results as described above.

6. An advantage of this method is the fact that it allows a rough quantitative estimation of albumin from the volume of precipitate after standing overnight. Complete solidification amounts to 2 to 3 per cent albumin. Precipitates reaching one-half, one-third, one-fourth and one-tenth the height of the column of urine correspond roughly to about 1, 0.5, 0.25 and 0.1 per cent albumin.

7. Certain resinous acids may be precipitated by the acid but these may be easily differentiated from albumin precipitate by reason of their solubility in alcohol.

STANDARDS FOR THE PURDY AND HEAT AND ACID TESTS

Fairly permanent standards, showing the clouds and precipitates but not the rings, may be prepared as follows and kept on hand for rapid comparison and more accurate readings. These will be found especially helpful for the Purdy and heat acid tests:

1. Dissolve 20 grams of gelatin in 100 c.c. of hot water.
2. Add 0.3 c.c. of formalin. Mix well. Keep at about 50° C.
3. In six thin-walled test tubes of the kind used for the heat and acid test, place the following amounts of serum:

No. 1: no serum	No. 4: 0.1 c.c. undiluted
No. 2: 0.2 c.c. of 1:10	No. 5: 0.3 c.c. undiluted
No. 3: 0.5 c.c. of 1:10	No. 6: 0.6 c.c. undiluted

4 To each tube add enough water to make 2 c.c.
5. To each tube add 8 c.c. of hot formalized gelatin.
6. Mix well by inverting several times.

7. Heat the upper third of each tube in a Bunsen to coagulate the albumin.
8. Cork and label each as follows:

> No. 1: negative —
> No. 2: very slight trace ($\pm$)
> No. 3: slight trace (+)
> No. 4: moderate trace (+ +)
> No. 5: heavy cloud (+ + +)
> No. 6: very heavy cloud (+ + + +)

Roberts' Test.—1. The test may be carried out by contacting reagent with urine in any of the following ways:

(*a*) Place a few c.c. of the reagent in a conical glass or test tube. Tilt and run clear urine from a pipet or medicine dropper down the side to give a sharp line of contact.

REAGENT

Magnesium sulphate (sat. aq. sol.) .... 5 parts
Nitric acid (conc.) .... 1 part

A saturated solution of magnesium sulphate is prepared by dissolving 100 grams in 80 c.c. of water at 25° C.

(*b*) Place urine in a horismascope and underlay with reagent as shown in Figure 103-(B). This instrument is too fragile and too expensive for general use although very handy for office work.

(*c*) Or immerse a pipet in the urine, wipe off the outside and immerse in the reagent, as shown in Figure 103-(A), according to Boston's method. Not as sensitive as (*a*) *and* (*b*).

2. If albumin is present, a white ring appears at the line of contact, best seen against a black background at a distance of several feet.

3. Record as described above.

4. This test is much more satisfactory than the Heller ring test employing nitric acid, as it is sensitive and does not form the secondary and confusing colored rings due to indican, bile pigments, or the oxidation of other organic constituents. It may, however, yield the secondary ring due to uric acid and urates and especially if the test is conducted in a conical glass. This ring is less sharply defined, broader and frequently situated above the albumin ring. Concentrated urines may occasionally exhibit a crystalline ring of urea nitrate, but this is easily distinguished from the "fluffy" ring of albumin.

QUANTITATIVE ESTIMATION OF ALBUMIN

Method of Kingsbury, Clark, Williams and Post.—This method, which has been adopted by the Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America, is based upon the *principle* that the

turbidity produced in urine by sulphosalicylic acid is compared with permanent standards representing known amounts of protein.

The reagent is a 3 per cent solution of sulphosalicylic acid in distilled water. To 2.5 c.c. of clear centrifuged urine in a test tube graduated at 10 c.c., add 3 per cent sulphosalicylic acid to the mark. Invert the tube to mix, let stand 5 to 10 minutes and compare the turbidity with the permanent standards.

Record the albumin content according to the standard most closely matched. For values above 100 mgm. per 100 c.c., repeat the test on a portion of urine
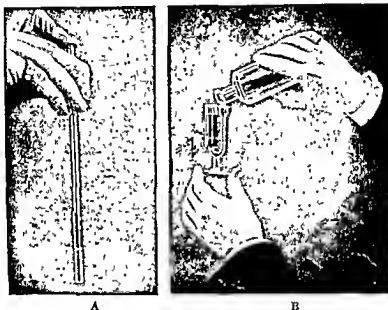
FIG. 103.—RING OR CONTACT TEST

A. Boston's method. *B.* Horismascope method. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co.)

sufficiently diluted with water to come within the range of the standards, multiplying the result obtained by the dilution.

Preparation of Permanent Standards.—Mark a 250 c.c. Erlenmeyer flask at 200 c.c. with a china marking pencil. Place exactly 20 gms. of very pure gelatine in this flask, add about 150 c.c. of water and warm at 45 to 55° C. until dissolved. Make up to the mark with water. Now add about one-half the white of an egg, mix thoroughly and heat in boiling water for 30 minutes. Filter hot through a Whatman No. 4 fluted filter paper. The filtrate should be perfectly clear though slightly yellowish. Immediately before using 0.3 c.c. of 40 per cent formalin is added to each 100 c.c. of filtrate.

Formazin, the material to be suspended in this solution, is prepared as follows: In a 50 c.c. graduated flask is placed 0.25 gm. of hydrazine sulphate and dissolved in 25 c.c. of water. To this solution is added a solution of 2.5 gms. of urotropin in

about 15 c.c. of water and water added to the mark. After standing about 15 hours a quantity of white amorphous precipitate will have formed. This may be uniformly distributed throughout the flask by gently inverting several times and while thus distributed may be accurately pipeted.

14.5 c.c. of this suspension is added to 100 c.c. of the clarified gelatin solution at 45-55° C. to which has just previously been added 0.3 c.c. of 40 per cent formalin and the whole thoroughly mixed. This produces a turbidity equivalent to 100 mgm. of albumin per 100 c.c. of urine when treated as in the above method; and from this 100 mgm. standard are prepared standards equivalent to 75, 50, 40, 30, 20 and 10 mgm. per 100 c.c. by diluting the 100 mgm. standard with the proper amounts of clarified gelatin to which formalin has also been added in the proportion of 0.3 c.c. per 100 c.c. as follows:

Value of Std. Desired; Mgm. per 100 c.c.	Amt. of 100 mgm. Std. used; c.c.	Amt. of 10% clarified Gelatine used; c.c.
75	7.5	2.5
50	5.0	5.0
40	4.0	6.0
30	3.0	7.0
20	2.0	8.0
10	1.0	9.0

These amounts are placed in test tubes of the same dimensions as those used in making the tests with urine. The tubes are stoppered and sealed with wax and allowed to cool to room temperature. After a few hours the gelatine will solidify after which it will remain hard and will not melt at any room temperature. Such standards keep for about a year. They gradually weaken and it is unwise to use them after about 10-12 months.

Exton's Quantitative Method.—1. Place 3 c.c. of urine in a test tube and add 3 c.c. of reagent. The tube in which the test is made should have the same dimensions as the standard tubes.

REAGENT

Sulphosalicylic acid (Eastman) .... 50 gms.
Sodium sulphate (crystals) .... 10 gms.
Bromphenol blue (0.4% watery solution) .. 25 c.c.

Dissolve the acid and sodium sulphate in about 800 c.c. of water and add the dye. Make up to 1000 c.c. and filter through acid washed paper (Whatman No. 0).

2. *Heat gently. It is not necessary to boil.*

(The purpose of the application of moderate heat is to increase the specificity of the reaction, as resinous bodies and other substances which might react go into solution when warmed. Any turbidity remaining is due to abnormal protein substances.)

3. Invert the standard tubes in order to secure a homogeneous suspension.

4. Read the turbidity of the unknown by comparing it with the nearest higher and lower standards[2] placed on either side.

If necessary take the reading between the two tubes.

If the urine contains more than 100 mg. per cent, and hence is cloudier than the highest standard tubes, dilute the mixture of urine and reagent with the diluting solution given below. Make another reading and multiply the reading by the dilution.

DILUTING SOLUTION

Sodium sulphate .... 10 gms.
Concentrated sulphuric acid..................... 5 c.c.
Bromphenol blue (0.4% watery solution)......... 25 c.c.
Water (distilled) .... q.s. 1000 c.c.

Dissolve the sodium sulphate in about 800 c.c. of water, then add the acid and bromphenol blue. Make up to 1000 c.c.

To make the test on cloudy urines which cannot be cleared, dilute the urine with an equal volume of diluting solution and read against the standard tubes. Precipitate a similar volume of urine in the usual way and read. The difference between the reading due to turbidity and that after precipitation represents the amount of albumin.

Urines containing large amounts of carbonates or other alkaline salts must be made acid before testing, in order to prevent frothing.

Because bromphenol blue is also an indicator on the alkaline side, to prevent decomposed or alkaline urines from turning purple, they must be made acid before testing.

If the color is very dark or unusual, the dilutions must be made with the diluting solution.

If a precipitate resembling curdled milk appears when the reagent is added, the urine contains Bence-Jones protein. This may be proved by the *disappearance* of the precipitate on boiling and its *reappearance* as the solution cools again.

Esbach's Method.—1. Fill with urine an Esbach-Quick albuminometer (Fig. 104) to the mark *U*.

2. Add reagent to the mark *R*:

REAGENT

Trichloracetic acid .... 100 gm.
Water .... 900 c.c.

3. Close with a rubber stopper, invert slowly several times, and set aside in a cool place for eighteen to twenty-four hours.

[2] Standards and reagents for Exton's quantitative method may be obtained from the Standard Reagents Co., Philadelphia, Pa.

4. Read off the results according to the markings on the tube which show albumin in grams per 1000 c.c.: to express the per cent, divide by 10.

5. The advantage of the new reagent over the former one of picric and citric acids is that the effects of temperature and specific gravity of the urine are reduced to a minimum. The latter reagent is, however, preferred by some and is prepared as follows:

Picric acid .... 10 gm.
Citric acid .... 20 gm.
Water .... 1000 c.c.

Tsuchiya's Method.—This method is generally regarded as superior to that of Esbach. If the urine is alkaline, acidify with a few drops of acetic acid and proceed in the same manner as in the Esbach method, using the following reagent:

REAGENT

Phosphotungstic acid .... 1.5 gm.
Hydrochloric acid (conc.) .... 5.0 c.c.
Alcohol (96 per cent) .... 95.0 c.c.

Fig. 104.—Esbach Quick Albuminometer

Folin's Method.—1. Place 1 c.c. of urine in a test tube and add a 2 per cent solution of sulphosalicylic acid to exactly 25 c.c.

2. In four additional tubes of the same size place 1 c.c. of 0.1, 0.2. 0.3, and 0.4 per cent solutions of a stock albumin solution and add the reagent to 25 c.c.

3. Mix and let stand ten to fifteen minutes and compare.

4. A stock albumin solution of approximately 1 per cent strength is made by diluting sheep serum with 7 volumes of 15 per cent sodium chloride solution. This keeps well, and the other standards are made from it by dilution with 15 per cent sodium chloride solution.

Method of Shevky and Stafford.[3]—*Principle.*—The quantity of protein is calculated from the volume occupied by the precipitate obtained with phosphotungstic acid, and alcohol.

Reagent.—Tsuchiya's reagent.

Procedure.—If the urine contains less than 0.28% protein it may be used without preliminary dilution. Nephritic urines are usually diluted 1:10 or 1:5. If the protein content is over 2.8%, it will be necessary to employ a 1:20 solution of the reagent.

1. Place 4 c.c. of urine as prepared above in the specially formed and graduated tube (A.H.T. cat. No. 3007-A) designed for this procedure.

2. Add the reagent to the 6.5 c.c. mark.

3. Close the tube and mix by slow inversion 3 times.

4. Allow to stand exactly 10 minutes.

5. Centrifuge for exactly 10 minutes at 1800 r.p.m.

6. Read the volume of the precipitate directly.

Calculation.—Grams of protein per liter = c.c. precipitate x 7.2 x dilution of urine.

This method possesses the great advantages of ease of performance and sufficient accuracy, if care is observed, for all clinical purposes. It is the method of choice for routine purposes in many laboratories.

DETECTION OF PROTEOSES

Principles.—Proteoses, particularly deuteroproteose and heteroproteose, have frequently been found in the urine under various pathological conditions. They are divided into two groups, namely, *primary* and *secondary*. The *primary proteoses* are precipitated upon half saturation with ammonium sulphate and the *secondary proteoses* upon complete saturation.

Procedure.—1. Acidify the urine with acetic acid and filter off any precipitate of nucleoprotein which may form.

2. Boil for several minutes.

3. Filter while hot to remove the albumin and globulin.

4. Test the filtrate by overlaying a saturated solution of trichloracetic acid. A white ring at the point of contact indicates the presence of proteoses.

5. If the test is positive, the primary and secondary proteoses may be separated by half and complete saturation with ammonium sulphate.

DETECTION OF BENCE-JONES PROTEIN

1. Place the urine in a water bath with a thermometer and heat very slowly and gently.

2. Observe frequently. Turbidity will begin to occur at about 40° C. and precipitation will take place at about 60° C.

3. Now acidulate *very slightly* with acetic acid and raise the temperature to the boiling point (100° C.). The precipitate now partly or totally disappears.

4. Allow to cool and if Bence-Jones protein is present the precipitate will reappear.

5. Urine containing albumin should be filtered at or near the boiling point, the aforementioned procedures being applied to the filtrate.

6. If the test is positive it is advisable to confirm the results by one or both of the following tests:

(a) Precipitate the protein with nitric acid. This precipitate should disappear on boiling and reappear upon cooling.

(b) Precipitate the protein with alcohol and collect immediately by centrifuging. The precipitate should be soluble in water.

DETERMINATION OF UREA (VOLUMETRIC)

Procedure (Van Slyke and Cullen's Modification of Marshall's Method).—
1. Measure the specimen and note whether it is a twenty-four hour specimen or a specimen for urea tolerance test. Make a determination of free ammonia (see below) at the same time this determination is run.

2. Pipet 5 c.c. urine into a 100 c.c. volumetric flask and dilute to mark.

3. With a pipet transfer 5 c.c. of the diluted urine to a thick-walled, large Pyrex test tube (200 by 25 millimeters).

4. Add two powdered urease tablets (25 milligrams each, Hynson, Westcott and Dunning).

5. Stopper and let stand at room temperature for one-half hour.

6. Add 2 drops of caprylic alcohol.

7. Add 5 c.c. of saturated potassium carbonate solution.

8. Draw off the ammonia by suction aeration (Fig. 105) into another tube containing 20 c.c. N/100 hydrochloric acid and 2 drops of methyl red indicator. Aeration should last about one hour and during the first two minutes only a slow current of air should be used.

Fig. 105.—Aeration Apparatus

9. Titrate the residue of acid with N/100 sodium hydroxide.

Calculation.—Each c.c. of acid neutralized indicates 0.00014 gram of nitrogen.

$$\left(20 - \text{c.c.} \frac{N}{100} \text{sodium hydroxide}\right) \times 4 \times 0.00014 \times \text{volume of urine in c.c.} = \text{grams urea nitrogen} + \text{ammonia nitrogen}$$

or

$$\left(20 - \text{c.c.} \frac{N}{100} \text{sodium hydroxide}\right) \times 0.00056 \times \text{volume of urine in c.c.} = \text{grams urea nitrogen} + \text{ammonia nitrogen}$$

(Grams urea nitrogen + ammonia nitrogen) — grams ammonia nitgoren = grams urea nitrogen.

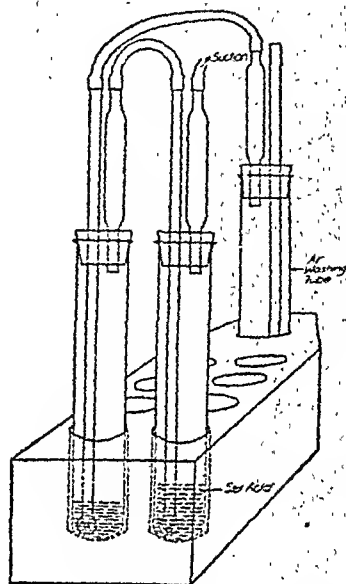Grams urea nitrogen $\times$ 2.145 = grams urea.

DETERMINATION OF UREA

Principle.—Urine is treated with permutit to remove ammonia, filtered, and the filtrate is used for the urea determination by means of Karr's method for urea nitrogen.

Calculation.—Set the urine sample at 15 mm. in the colorimeter, when the reading of the standard multiplied by 0.02 = grams of total nitrogen per 100 c.c.

$$\text{Grams total nitrogen per 100 c.c.} \times \frac{\text{c.c. volume of 24-hour specimen}}{100}$$

= grams total urinary nitrogen per twenty-four hours

DETECTION OF DEXTROSE (GLUCOSE)

Principles.—1. Dextrose or glucose readily reduces the oxide of copper in alkaline solution. When the whitish-blue cupric hydroxide in suspension in an alkaline solution is heated it is converted into insoluble black cupric oxide, but if sugar is present this is reduced to insoluble yellow or red cuprous oxide.

2. A large number of tests have been devised on this principle for the detection of sugar in the urine but that of Benedict is recommended because of its sensitiveness, simplicity, and freedom from error. The qualitative reagent does not react with the normal sugar of the urine but detects increases above this level as low as 0.25%. Furthermore, uric acid, creatinine, chloroform, formalin and other aldehydes do not interfere to such an extent as in the case of Fehling's test.

3. If albumin is present in large amounts, it may interfere with the precipitation of copper and should be removed by acidifying with acetic acid, boiling and filtering. Small amounts need not be removed.

Benedict's Test.—1. Place 5 c.c. of Benedict's *qualitative* reagent in a clean test tube.

BENEDICT'S QUALITATIVE REAGENT

Copper sulphate	17.3 gm.
Sodium citrate	173.0 gm.
Sodium carbonate (anhydrous)	100.0 gm.
Distilled water to make	1000.0 c.c.

Dissolve the citrate and carbonate in about 500 c.c. of distilled water by heating. Dissolve the copper sulphate in about 100 c.c. of water.

Add the copper solution slowly to the citrate and carbonate solution and stir continuously while adding.

Measure and add sufficient water to make the total volume 1000 c.c. *Do not use for quantitative test.*

Filter through paper.

2. Add 0.5 c.c. of urine and mix thoroughly.

3. Boil thoroughly for 2½ to 5 minutes; or place tubes in a boiling water bath for five minutes—a particularly convenient method when conducting a large number of tests at one time.

4. Allow to cool spontaneously.

5. If no sugar is present the solution will remain clear or show only a slight turbidity of a faint bluish color due to urates. If sugar is present a green, red, or

yellow precipitate will form. the color depending upon the amount of sugar present.

6. Even 0.25 per cent glucose yields a large bulk of precipitate, filling the solution and rendering it opaque so that the test may be applied as readily in artificial light as in daylight.

7. The following scheme may be used for reporting (after Todd and Sanford):

+ (1) = *slight trace*. No reduction is evident during boiling but appears upon cooling (greenish).

++ (2) = *trace*. Reduction occurs after about one minute's boiling.

+++ (3) = *moderate*. Reduction occurs after ten to fifteen seconds' boiling.

++++ (4) = *large amount*. Reduction occurs almost immediately after adding urine to the boiling reagent.

Fermentation Test.—1.   Place 15 c.c. of urine in a test tube and add a piece of fresh Fleischman yeast about the size of a pea; mix gently to emulsify the yeast.

2. Transfer to a fermentation tube; make sure the arm is free of bubbles of air.

3. Place in an incubator for a few hours.

4. A normal urine and a normal urine to which is added a pinch of glucose may be treated in the same manner as negative and positive controls respectively.

5. A positive reaction due to alcoholic fermentation is indicated by the collection of carbon dioxide gas in the arm.

6. If necessary, guard against gas production by bacterial fermentation by adding a pinch of tartaric acid (advisable if mixtures are incubated more than four hours).

FIG. 106.—EINHORN SACCHAROMETER

7. By using the Einhorn saccharometer (Fig. 106), a *quantitative* test may be conducted, as the graduations on the arm indicate with fair accuracy the percentage of glucose present from 0.1 to 1%.

QUANTITATIVE ESTIMATION OF DEXTROSE

Benedict's Method.—1.   Dilute 10 c.c. of urine with 90 c.c. of water.

2. Mix and place in a buret (Fig. 107).

3. Place 25 c.c. of Benedict's quantitative reagent in a porcelain evaporating dish.

BENEDICT'S QUANTITATIVE REAGENT

Copper sulphate (pure crystallized)	18 gm.
Sodium carbonate (crystallized)	200 gm.
(*or* anhydrous sodium carbonate..100 gm.)	
Sodium or potassium citrate (C.P.)	200 gm.
Potassium sulphocyanate (C.P.)	125 gm.
Potassium ferrocyanide solution (5 per cent) ...	5 c.c.
Distilled water to make.....................	1000 c.c.

With the aid of heat dissolve the carbonate, citrate and sulphocyanate in about 700 c.c. of the water and filter. Dissolve the copper in 100 c.c. of water and pour slowly in the other, stirring constantly. Add the ferrocyanide solution, cool and bring up to 1000 c.c. with distilled water. It keeps well.

4. Add to the reagent 10 grams of sodium carbonate crystals and a little pumice, talc or a few glass beads.

5. Apply heat to reagent until boiling.

6. While the reagent is boiling, run in the urine a little at a time but fairly rapidly, until a chalk-white precipitate appears and the blue color of the reagent begins to fade. Then add urine a drop or two at a time until all color disappears.

7. Note the amount of diluted urine used.

8. The reagent is so prepared that 25 c.c. are reduced by 0.05 gram of glucose. Therefore the amount of diluted urine used contains this amount.

9. Divide the amount of diluted urine by 10 to give the amount of undiluted urine carrying 0.05 gram of glucose. Divide 0.05 by the number of c.c. of undiluted urine to obtain the amount of sugar contained in 1 c.c. of urine; then multiply this number by 100 to obtain percentage or by the total number of c.c. in the twenty-four hour specimen to obtain the number of grams voided. Example:

Fig. 107.—QUANTITATIVE ESTIMATION OF SUGAR IN URINE

2180 c.c. of urine voided in twenty-four hours
8.2 c.c. urine 1:10 required to reduce 25 c.c. of reagent

$$\frac{8.2}{10} = 0.82 \text{ c.c. undiluted urine required}$$

$$\frac{0.05}{0.82} \times \frac{100}{1} = 6.1 \text{ per cent}$$

$$\frac{0.05}{0.82} \times \frac{2480}{1} = 151.2 \text{ gm. in twenty-four-hour specimen}$$

10. A short method to obtain the percentage is to divide 50 by the number of c.c. of diluted urine required to reduce 25 c.c. of the reagent. Example:

2480 c.c. voided in twenty-four hours
8.2 c.c. diluted urine required

$$\frac{50}{8.2} = 6.1 \text{ per cent.}$$

11. If the urine contains but a trace of sugar it should be used undiluted and the percentage calculated as above. Example:
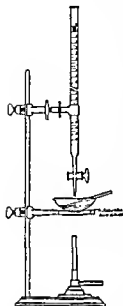
2870 c.c. voided in twenty-four hours
5.2 c.c. undiluted required

$$\frac{0.05}{5.2} \times \frac{100}{1} = 0.96 \text{ per cent}$$

Benedict's Test Tube Method.—1. Place 5 c.c. of Benedict's quantitative reagent in a clean test tube.

2. Add 1 or 2 grams of anhydrous sodium carbonate and an equal amount of pumice.

3. Heat to boiling over Bunsen burner.

4. While boiling run into tube from a 1 c.c. pipet, graduated in 0.1 c.c., undiluted urine until the last trace of blue has disappeared. The urine should be run in slowly and the solution kept boiling while the urine is being added.

5. As 5 c.c. of reagent are reduced by 0.010 gram of glucose, the amount of urine used contains this amount.

6. To obtain the percentage, divide 100 by the amount of urine and multiply by 0.010:

0.8 c.c. urine required

$$\frac{100}{0.8} \times \frac{0.010}{1} = 1.25 \text{ per cent}$$

7. Or divide 1 by the amount of urine to obtain the percentage:

$$\frac{1.0}{0.8} = 1.25 \text{ per cent}$$

8. If the urine contains a large amount of sugar, dilute 1:10 and calculate accordingly.

Benedict's Picric Acid Method.—This method, which has been adopted by the Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America, is based upon the *principle* that the color produced by the reduction of picric acid is compared with permanent standards which have colors equivalent to that produced by known amounts of glucose solutions.

Reagents: (1) Picric acid solution: Two grams of pure, dry picric acid are dissolved in one liter of water.

(2) 5 per cent sodium hydroxide solution.

(3) A 50 per cent solution of acetone prepared fresh each day by diluting a definite quantity of C.P. acetone with an equal part of water.

Procedure: (1) Measure 1 c.c. of urine into a test tube graduated at 25 c.c.

(2) Add 3 c.c. of the picric acid solution.

(3) Add 0.5 c.c. of the 5 per cent solution of sodium hydroxide.

(4) Add 5 drops of the 50 per cent acetone.

(5) Mix by sidewise shaking and place at once in boiling water.

(6) After 12 minutes remove the tube, cool and dilute to the 25 c.c. mark.

(7) Compare with the permanent standards,* which gives results directly as per cent glucose.

(8) If more than 0.5 per cent glucose is expected, dilute the urine with 9 or 19 parts of water and make the determination on 1 c.c. of the diluted specimen. Results are of course then multiplied by 10 or 20.

Preparation of Permanent Standards.—(1) Dissolve 100 gms. ferric chloride in about 150 c.c. of water. Add 2 drops of concentrated hydrochloric acid and transfer to a 250 c.c. volumetric flask. Dilute to the mark and mix. If not perfectly clear, filter through a dry filter into a dry flask.

(2) Dissolve 75 gms. cobalt chloride in about 15 c.c. water and transfer to a 250 c.c. volumetric flask. Add 2 drops of concentrated hydrochloric acid. Dilute to the mark and mix. Filter through dry paper into a dry flask.

(3) Dilute 5 c.c. of concentrated hydrochloric acid to 50 c.c. with water.

The above solutions are mixed according to the proportions given in the following table:

Glucose per cent	Ferric Chloride Solution c.c.	Cobalt Chloride Solution c.c.	Dilute HCl c.c.	Water c.c.
0.1	18	8	8	66
0.2	28	16	8	48
0.3	22	30	8	40
0.4	16	43	8	33
0.5	14	65	8	13

(4) Place about 25 c.c. of each standard in test tubes of the same diameter as those used for the glucose determination.

It is of course essential that the above colors be matched against glucose solutions of known strength and such adjustments made in the permanent standards as may be required to give the proper color since there may be some variation in color strength of the original reagents. The inorganic color standards keep practically indefinitely.

Sumner's Method.[a]—1. Into a small test tube, pipet 1 c.c. of the urine to be examined; add 9 c.c. of water with a pipet or by diluting to a 10 c.c. mark on the tube. Mix.

2. Pipet 1 c.c. of this diluted urine into a Folin sugar tube or to a test tube graduated at 25 c.c.

3. Add 3 c.c. of the reagent and place in boiling water for 5 minutes. Cool in running water, dilute to mark, and mix.

4. Compare with the standard tubes and read directly the percentage from the tube. In case it is stronger than 3 per cent, dilute with a definite proportion of water, generally equal parts, and multiply the answer by the dilution factor.

* These standards, in hermetically sealed tubes, may be obtained from the Standard Reagents Company, Philadelphia, Pa.

[a] *J. Biol. Chem.*, 1925, 65:373.

Notes.—1. Slightly increased accuracy may be obtained by using an artificial or glucose standard and the colorimeter.

2. In the above method the qualitative test may be combined with the quantitative. All urines reading 0.2% or under may be considered *negative* for glucose.

3. Sugar in normal urine may be read by using undiluted urine in the above test when the percentage of sugar will be that given on the standard tubes divided by 10.

4. Urines showing 0.25% or over will give a positive Benedict qualitative test.

Reagent.—Dissolve 10 grams of dinitrosalicylic acid in water and dilute to 1 liter (1 per cent).

Place 300 c.c. of 4.5 per cent sodium hydroxide in a 2 liter graduated cylinder; add 880 c.c. of 1 per cent dinitrosalicylic acid and 255 grams of sodium potassium tartrate. Mix until dissolved. Preserve in a brown bottle in the dark.

Permanent Standard Series.—Dissolve 0.8625 gram of ferric ammonium sulphate in water and dilute to 100 c.c. in a volumetric flask. Into each of twelve 100 c.c. volumetric flasks add 10 c.c. of the 1% dinitrosalicylic acid solution and add in order 1, 1.75, 2.53, 3.22, 4, 4.68, 5.32, 6.10, 6.80, 7.5, 9.1, 10.3 c.c. of ferric ammonium sulphate solution. Dilute each to 100 c.c. and mix. Transfer portions of each to tubes similar to those used in doing the test and label these tubes respectively, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.5, 3%. These tubes will then read directly in percentage of sugar considering the 1:10 dilution as made in the test.

DETECTION OF LEVULOSE

Principles.—Levulose (fructose) may appear in the urine after the ingestion of large quantities of honey and in severe diabetes, together with glucose.

Seliwanoff's Test.—1. Place 5 c.c. of reagent in a test tube and add a few drops of urine.

REAGENT

Hydrochloric acid	1 part
Distilled water	2 parts

2. Heat to boiling. Do not prolong the boiling.

3. If levulose is present a red color will develop with the separation of a red precipitate. The precipitate must be soluble in alcohol with a bright red color.

4. The urine for this test must not contain over 2% glucose.

Borchardt's Test.—1. Place 5 c.c. of urine in a test tube and add 5 c.c. of 25 per cent hydrochloric acid (conc. HCL, 2 parts; water, 1 part).

2. Add a few crystals of resorcinol.

3. Boil for not more than one-half minute. (If levulose is present a red color appears.)

4. Cool in running water, pour into a beaker and render slightly alkaline with solid sodium or potassium hydroxide.

5. Pour back into test tube, add 2 or 3 c.c. of acetic ether, and shake.

6. If levulose is present, the ether will be colored yellow.

The administration of rhubarb or senna will cause a yellowish coloration of the ether similar to levulose. If indican is present it should be removed by doing the Obermayer's test. After the chloroform has extracted the indican remove the supernatant fluid and reduce the acidity by adding one-third its volume of water. Proceed with the test omitting step I.

DETECTION OF GALACTOSE

Principles.—Galactose may occur in the urine of nursing infants with severe disturbance of the digestive tract. It may be differentiated from other reducing sugars (except lactose) by the formation of mucic acid.

Test.—1. Place 100 c.c. of urine in a broad, shallow, evaporating dish.

2. Add 20 c.c. of nitric acid and evaporate over boiling water until volume is about 20 c.c. (If the specific gravity of the urine is over 1.020 add 25 to 35 c.c. of acid and evaporate to a volume equal to the volume of acid used.)

3. If galactose (or lactose) is present the fluid will be clear with a fine white precipitate of mucic acid.

DETECTION OF LACTOSE

Principles.—Lactose is occasionally found in the urine of women during lactation and in patients who have been on an exclusive milk diet for a long time. It reduces copper solutions, although less actively than glucose, 0.0676 gm., being equivalent to 25 c.c. of Benedict's quantitative solution. It is not fermented by yeast.

Rubner's Test.—1. To 10 c.c. of urine add 3 grams of lead acetate (an excess).

2. Shake well and filter into a test tube.

3. Boil the filtrate for a few seconds; add 1 c.c. of strong ammonia and boil again.

4. If lactose is present, the solution turns brick-red and a red precipitate develops which is the criterion.

5. This test is not very sensitive but will detect lactose in about 0.3 to 0.5%.

6. Dextrose (glucose) gives a red solution with a yellow precipitate.

7. Lactose does not ferment with yeast although bacteria may hydrolyze it into its constituents, glucose and galactose.

DETECTION OF PENTOSE

Principles.—Pentosuria may be *alimentary* and temporary, because of the ingestion of large amounts of pentose-rich fruits, or pathological (especially in diabetes). The pentose detected most frequently in the chronic form is arabinose. The color reaction is based upon the production of furfurol.

Bial's Orcinol Test.—1. First remove the dextrose by fermentation (see page 147). Filter.

2. Place 5 c.c. of reagent in a test tube and heat to boiling.

REAGENT

Hydrochloric acid (30 percent) .... 500 c.c.
Ferric chloride solution (10 per cent) .... 1 c.c.
Orcinol .... 1.5 gm.

3. Remove from the flame and add the urine drop by drop (not exceeding 20 drops in all).

4. The appearance of a green color indicates presence of pentose.

5. This test is recommended because it is more accurate than the original orcinol test.

DETECTION OF ACETONE

Acetone, diacetic acid (aceto-acetic acid), and *b.-hydroxybutyric* acid are the three acetone bodies which occur in the urine. They practically always occur together. It is believed that the kidneys do not excrete acetone but do excrete diacetic acid, which decomposes readily into acetone in the urine. The tests for acetone are, therefore, probably tests for diacetic acid. Both acetone and diacetic acid respond to the nitroprusside tests. Acetone does not give a reaction in the ferric chloride test for diacetic acid.

Principles.—The detection of acetone is based upon a color reaction with nitroprusside (Rothera's test) in which there is the formation of ferropentacyanide with the isonitro compound of the ketone or the formation of such an ion with the isonitroanine derivative of the ketone. In the Frommer test the color is due to the formation of dihydroxydibenzoylacetone through the interaction of salicylaldehyde and acetone.

Tests for acetone may be unsatisfactory when applied directly to urine. By distilling the urine the acetone is obtained in purer form. Any diacetic acid in the urine will pass into the distillate as acetone. A simple apparatus for distilling small amounts of urine is shown in Figure 108.
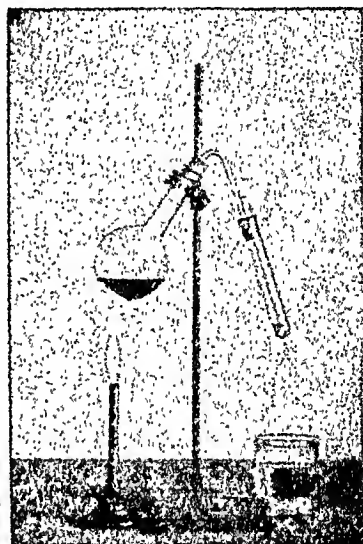

FIG. 108.—A SIMPLE DISTILLING APPARATUS

The longer the delivery tube the better will the vapor condense. Condensation may also be facilitated by immersing the test tube in a glass of cold water. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co.)

Rothera's Test.—1. To 5 or 10 c.c. of filtered or distilled urine add about 1 gram of ammonium sulphate.

2. Add 2 or 3 drops of a freshly prepared 5% solution of sodium nitroprusside.

3. Mix thoroughly.

4. Stratify with strong ammonium hydroxide.

5. If acetone is present, a permanganate color will develop at the line of contact.

A modification of this test is as follows:

1. Place 3 to 5 c.c. of urine in test tube.
2. Add 1 c.c. of reagent and mix thoroughly.

REAGENT

Ammonium nitrate	30 gm.
Sodium nitroprusside	2 gm.
Water (distilled)	80 c.c.

3. Overlay with strong ammonia water.

4. A purple or permanganate ring develops at the junction of the fluids if acetone or diacetic acid is present.

5. Diacetic acid, however, reacts quickly while acetone reacts more slowly and with less intense color, so that the two reactions grade into each other.

Frommer's Test.—1. To 10 c.c. of undistilled urine in a test tube add 3 c.c. of 40% solution of sodium hydroxide.

2. Add 10 drops of a 10% alcoholic solution of salicylaldehyde.

3. Mix and heat the upper portion to about 70° C. (do not boil) for 5 to 10 minutes.

4. In the presence of acetone an orange color changing to deep red appears in the heated portion. A yellow to brown color is negative.

Wallhauser's Test.—1. Place 1 drop of Scott-Wilson reagent on an ordinary microscopic slide and place it over the mouth of the urine container to form a hanging drop, taking care that the regent does not come in contact with the container.

REAGENT

Mercuric cyanide	1.0 gm.
Water	60.0 c.c.

Add a cooled solution of 18 grams of sodium hydroxide dissolved in 60 c.c. of water. Transfer to a heavy glass jar and add 0.29 gram of silver nitrate dissolved in 40 c.c. of water while constantly stirring. Will keep for six months in a tightly stoppered dark bottle.

2. Allow to stand for two minutes.

3. Examine macroscopically.

4. If the reagent remains clear, the test has given a negative reaction. If cloudy, or a precipitate forms, a positive reaction is indicated.

DETECTION OF DIACETIC ACID

Principle.—The detection of diacetic (aceto-acetic) acid depends upon the production of a bordeaux red or violet red color with a dilute solution of ferric chloride.

Gerhardt's Test.—1. To about half a test tube full of fresh urine add a 10 per cent ferric chloride solution drop by drop until the phosphates are precipitated.

2. Filter.

3. To the filtrate add more of the ferric chloride solution or place a small amount of the reagent in a test tube and carefully overlay with filtrate for a ring or contact test.

4. If diacetic acid is present the solution will turn a bordeaux red color, but since a similar color may result from the presence of phenol, salicylates. antipyrine, sodium bicarbonate and other substances. it is necessary to repeat as follows:

1. To 5 c.c. of urine in a test tube add 5 c.c. of water and boil down to 5 c.c.

2. After cooling, add the ferric chloride as above.

3. Since boiling drives off diacetic acid. the development of the color indicates that it is due to other substances. If doubtful. apply the following test.

Lindemann's Test.—1. To 10 c.c. of urine in a test tube add 5 drops of 30% acetic acid, 5 drops of Lugol's solution and 3 c.c. of chloroform.

2. Shake well and allow chloroform to settle.

3. If diacetic acid is present, the chloroform does not change color but becomes reddish-violet in its absence.

4. If the urine contains much uric acid. use double the amount of Lugol's solution.

DETECTION OF BETA-OXYBUTYRIC ACID

Hart's Test.—1. Dilute 20 c.c. of urine with an equal amount of water and add a few drops of acetic acid.

2. Reduce to one-half its volume by boiling to remove acetone and diacetic acid.

3. Dilute to 20 c.c. with water and place 10 c.c. in each of two test tubes.

4. To one tube add 1 c.c. of hydrogen peroxide and warm gently for one minute. Then allow to cool.

5. To both tubes add 10 drops of glacial acetic acid and 10 drops of freshly prepared concentrated sodium nitroprusside solution.

6. Mix thoroughly.

7. Overlay with strong ammonia water.

8. Allow to stand three or four hours.

9. A positive result is a purple ring in the tube treated with the peroxide, and none in the other.

DETECTION OF INDICAN

Principle.—The detection of indican (indoxyl potassium sulphate) by the test given below depends upon its decomposition and subsequent oxidation of the indoxyl set free into indigo blue and its absorption by chloroform.

Obermayer's Test.—1. Add to about 5 c.c of urine in a test tube an equal volume of Obermayer's reagent and mix thoroughly.

REAGENT

Ferric chloride .... 2 gm.
Hydrochloric acid (conc. sp. gr. 1.19) ..... 1000 c.c.

2. Heat until tube is warm.

3. Add 2 c.c. of chloroform and mix thoroughly by inverting, but avoid violent shaking.

4. Allow chloroform to settle.

5. If indican is present, the chloroform will be colored blue, ranging from a trace to a very deep blue, depending upon the amount present. The indican in normal urine may give a faint blue.

6. The urine of patients taking iodides may give a reddish-violet color which may obscure an indican reaction. By adding a few drops of concentrated sodium hyposulphite solution and shaking, the violet color will disappear, leaving the blue if indican be present. Occasionally, owing to slow oxidation, indigo red will form instead of indigo blue. This resembles the color given by iodides but does not disappear when treated with sodium hyposulphite.

7. Hexamethylenamine (urotropin) when taken by patients may prevent the reaction as likewise when it or formalin are added to urine as preservatives.

DETECTION OF BILE PIGMENTS

Principle.—The tests given below depend upon the oxidation of bile pigments by acids with the formation of a series of colored derivatives like biliverdin (green), bilicyanine (blue) and choletelin (yellow). Bilirubin is perhaps the most important pigment.

Rosenbach's Modification of Gmelin's Test.—1. Filter 100 c.c. or more of urine through a small filter paper.

2. Remove the filter paper from the funnel and allow it to partially dry.

3. Touch the paper with a drop of old or yellow nitric acid.

4. If bile is present, a most marked spreading ring of rainbow colors with green on the outside will form.

Huppert's Test for Bilirubin.—1. To 10 or 15 c.c. of urine add a saturated solution of calcium chloride.

2. Filter.

3. Discard filtrate, and after perforating the filter wash precipitate into beaker with a small amount of alcohol acidulated with sulphuric acid.

4. Boil the solution.

5. If bilirubin is present, the solution will assume a bright emerald green color.

Naumann Method.[5]—This procedure has been found to be much more sensitive than the preceding.

Principle.—The urinary pigments are adsorbed on a layer of talc and bili-cyanine is produced by oxidation of bilirubin by Fouché's reagent or nitric acid.

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[5] *Biochem. J.*, 1936, 30:762.

Reagents.—1. 10% aqueous suspension of talc.

2. Fouché's reagent: ,

Trichloracetic acid	25 gm.
Ferric chloride (10%)	10 c.c.
Distilled water	100 c.c.

3. 10% nitric acid.

Procedure.—1. Place a piece of wet filter paper (3 cm. diameter) in a Büchner filter (3.5 cm. diameter). Introduce 5 c.c. of the talc suspension after it is well shaken.

2. Dry by suction and pour 5 c.c. of urine over the layer of talc.

3. Dry by suction, place one drop of either Fouché's reagent or 10% HNO_3 on center of talc disc and dry by suction.

4. The presence of even traces of bilirubin is indicated by the appearance of a distinct blue color which increases in intensity for 1-2 hours and then fades slowly.

Note: Bilirubin has been detected in normal urine by this method in amounts of about 0.3 mg. per 100 c.c.

DETECTION OF BILE ACIDS

Principles.—In Hay's test, advantage is taken of the fact that bile acids have the property of reducing the surface tension of fluids in which they are contained. Oliver's test depends upon the principle that a precipitate is formed of a protein (peptone) and bile acids.

Hay's Test.—1. Cool the urine by placing it in a refrigerator for several hours.

2. Upon the surface sprinkle a little finely powdered sulphur ("flowers of sulphur").

3. If the sulphur sinks at once, bile acids are present to the amount of 0.01% or more. If the sulphur sinks only after gentle agitation, bile acids are present in 0.0025% or more. If the sulphur remains floating, even after gentle shaking, bile acids are absent. Chloroform and turpentine give false positive reactions.

DETECTION OF BILE SALTS

Oliver's Test.—1. Filter a small amount of urine (5 or 10 c.c.) until perfectly clear.

2. Acidify with acetic acid (not necessary if already acid).

3. Dilute with water until specific gravity is less than 1.008.

4. Place 2 c.c. in test tube.

5. Add 5 c.c. of reagent.

REAGENT

Peptone	8.33 gm.
Salicylic acid	1.12 gm.
Water containing 2 drops of acetic acid..	1000.00 c.c.

6. A positive reaction is indicated by a milky turbidity, which disappears on shaking, but reappears when more of the reagent is added. The presence of thymol vitiates the test. A positive reaction may also be obtained in the presence of chloroform or after the administration of turpentine or its derivatives.

If the color remains a light red within the time allowed, the reaction indicates normal value. If the original reaction indicates an increase then the following quantitative test may be conducted. With a little experience one can judge by the color whether there is an increase above normal.

DETECTION OF UROROSEIN

Test for Urorosein.—This pigment does not occur in normal urine but may occur in various diseases, such as pulmonary tuberculosis, typhoid fever, nephritis, and stomach disorders. It is excreted as a chromogen (indole acetic acid) which is transformed into the pigment upon treatment with a mineral acid:

1. Place 10 c.c. of urine in a test tube.
2. Add 5 c.c. of concentrated hydrochloric acid and a few drops of a 1% solution of potassium nitrite.
3. Mix thoroughly.
4. A rose-red color indicates urorosein.

DETECTION OF UROBILIN AND UROBILINOGEN

Schlesinger's Test for Urobilin.—It is claimed that urobilin is excreted as a chromogen, *urobilinogen*, which is changed by light into urobilin within a few hours after the urine is voided.

1. To 10 c.c. of urine in a test tube add a few drops of Lugol's solution to transform the chromogen into the pigment.
2. Add 10 c.c. of a saturated alcoholic solution of zinc acetate.
3. Mix and filter.
4. View the filtrate in sunlight against a dark background or with light concentrated upon it with a lens; a greenish fluorescence indicates the presence of urobilin.
5. Bile pigment, if present, should be previously removed by adding one-fifth volume of 10% solution of calcium chloride and filtering.

Ehrlich's Test for Urobilinogen (Wallace and Diamond).[a]—Urobilinogen is a normal constituent and, as stated above, is converted into urobilin upon standing. It is increased whenever there is an excess of bilirubin formed through excessive destruction of erythrocytes, especially in pernicious anemia and malaria. When the liver cells fail to function properly there is an increase and for this reason the test has become of practical value as a *liver function test.*

A marked decrease or even total absence may occur in obstructive jaundice when the obstruction is complete or nearly complete. If the obstruction is only partial it may be normal or even increased.

1. Place 10 c.c. of urine in a test tube 15 millimeters in diameter. The urine

[a] *Arch. Int. Med*, 1925, 35:698.

should not be too cold. If very cold, allow it to stand at room temperature or gently heat to 65° F. to 80° F.

2. Add 1 c.c. of Ehrlich's reagent and mix.

REAGENT

Paradimethylaminobenzaldehyde	2 gm.
Hydrochloric acid (conc.)	50 c.c.
Water	50 c.c.

3. Allow to stand for from one to three minutes, at the end of which time a cherry-red color appears if urobilin is present in abnormal amount. A light red and shades of pink appear when it is present in normal quantity. The examination for color should be made by viewing the contents through the mouth of the tube, holding it at a slight angle over white paper.

4. If no color appears the tube should be allowed to stand longer and then if still no color appears it should be heated and again examined before reporting an absence of urobilinogen.

5. A *quantitative* test may be conducted as follows:

(a) Place 6 test tubes (15 millimeters in diameter) in a rack. In them place the following amounts of urine:

No. 1...............	10 c.c.	1:20	No. 4...............	10 c.c.	1:50
No. 2...............	10 c.c.	1:30	No. 5...............	10 c.c.	1:100
No. 3...............	10 c.c.	1:40	No. 6...............	10 c.c.	1:200

The water used for diluting should not be too cold. If tap water is used it may be necessary to add enough warm water to bring it to about room temperature (between 65° F. and 85° F.)

(b) To each tube add 1 c.c. of Ehrlich's reagent and mix.

(c) At the end of *five minutes* examine the tubes by viewing through the mouth against a white background. Note the highest dilution which shows the slightest pink color. This is reported as positive 1:50, 1:200, etc.

When possible the readings should be made by daylight, as artificial light has a tendency to intensify the color. Highly concentrated urines may give a yellowish brown discoloration which has to be differentiated from the true pink reaction of urobilinogen. Readings up to 1:20 are considered normal.

(d) Bile pigment, if present, should be previously removed by adding 1 part of 10% solution of calcium chloride to 4 parts of urine, and filtering.

(e) Pyridium if present, will give a positive reaction to the test. Therefore specimens containing this drug are unsuitable for testing. This false positive reaction may be detected by treating the urine with HCl alone. A pink or red color with acid indicates the presence of the dye. By discontinuing the pyridium for three or four days a true test for urobilinogen can be obtained. Schlesinger's test for urobilin described on page 158, is not affected by pyridium and may be substituted in such cases.

(*j*) Wallace and Diamond recommend the examination of single fresh specimens rather than total twenty-four specimens, as at least one specimen with urobilinogen content may be found. Such increase, if occurring even once a day, signifies a pathologic condition.

EHRLICH'S DIAZO REACTION

The exact nature of the diazo substance or substances is unknown. It may be due to an increased excretion of urochromogen, alloxyproteic acid, oxyproteic acid, or uroferric acid. The reaction occurs in the urine in febrile disorders, especially typhoid fever, tuberculosis, and measles. Reactions more or less resembling it may occur after the administration of opium and its alkaloids, salol, creosote, phenol, the iodides, naphthaline and tannic acid.

The reagents are:

No. 1

Sulphanilic acid	1.0 gm.
Hydrochloric acid (conc.)	10.0 c.c.
Water	200.0 c.c.

No. 2

Sodium nitrite	0.5 gm.
Water	100.0 c.c.

No. 3

Strong ammonia water

1. Mix 10 c.c. of No. 1 with 0.1 c.c of No. 2 in a test tube (Greene).
2. Mix and add an equal amount of urine.
3. Mix and carefully overlay with 1 or 2 c.c. of No. 3.
4. A positive reaction is indicated by a garnet (eosin pink to deep crimson) red color at the line of contact. Upon shaking a distinct pink color is imparted to the foam (essential feature). The color is a pure pink or red; any trace of yellow or orange is a negative reaction. A doubtful reaction should be considered negative.

DETECTION OF UROCHROMOGEN

Principles.—According to Weisz, the detection of urochromogen in the urine is of importance in tuberculosis. He believed the diazo reaction to be due to urochromogen which fails to be converted into urochrome. The permanganate reaction detects both urochromogen and an antecedent substance. It has about the same value as the diazo reaction.

Weisz's Test.[7]—1. To 3 c.c. of urine in a test tube, add 2 volumes of water and divide the diluted urine between 2 tubes, one serving as a control.

2. To one tube add 3 drops of a 1:1000 aqueous solution of potassium permanganate.

[7] *München. Med. Wchnschr.*, 1911, 58:1348.

3. Shake well. A yellow color or a deepening of the color when compared with the control indicates urochromogen.

DETECTION OF BLOOD ⅄

Principles.—The conditions in which blood occurs in the urine may be classified under *hematuria* and *hemoglobinuria*. In the former one is able to detect not only hemoglobin but the unruptured corpuscles as well (see microscopy). whereas in the latter the hemoglobin alone is present.

The presence of blood is usually detected by the color of the urine. but the detection of traces requires microscopical and chemical examination. For the latter the usual tests for "occult blood" are required.

Benzidine Test.—1. Prepare a saturated solution of benzidine base (Merck's) by dissolving a knife point full in 2 c.c. of glacial acetic acid in a test tube. Warm if necessary.

2. Add an equal volume of 3% hydrogen peroxide.

3. Add 2 c.c. of the urine and mix.

4. The appearance of a blue color indicates a positive reaction.

5. Set up a control, using water instead of urine.

As the reaction with urine may be sufficiently turbid to mask the color. the following test by Ruttan and Hardisty is recommended as being more satisfactory:

Orthotoluidine Test.—1. In a test tube mix 1 c.c. of reagent with 1 c.c. of urine and 1 c.c. of 3% hydrogen peroxide.

REAGENT

Orthotoluidine .... 4.0 c.c.
Glacial acetic acid q.s. .... 100.0 c.c.

Dissolve. Keeps for a month without loss of delicacy.

2. In the presence of blood a bluish color develops (sometimes rather slowly) which persists for some time (several hours in some instances).

DETECTION OF HEMATOPORPHYRIN

Principle.—The pigment is precipitated with the phosphates. recovered, washed and prepared in solution in acid alcohol for spectroscopic examination.

Procedure.—1. To 100 c.c. of urine add 20 c.c. of a 10 per cent solution of sodium hydroxide.

2. Filter or centrifugalize off the precipitate.

3. Wash the precipitate with water and with alcohol.

4. Add 5 c.c. of alcohol and 5 to 10 drops of concentrated hydrochloric acid.

5. Dissolve, filter until absolutely clear and examine spectroscopically for the absorption bands of acid hematoporphyrin (Fig. 109).

6. An acetic acid test, which is much less reliable, consists in adding 5 c.c. of glacial acetic acid to 100 c.c. of urine and allowing the mixture to stand forty-eight hours. The pigment deposits in the form of a precipitate.

DETERMINATION OF CHLORIDES

Principle.—The principle of the Volhard method is employed; precipitation of silver chloride with a known amount of silver nitrate and titration of the excess
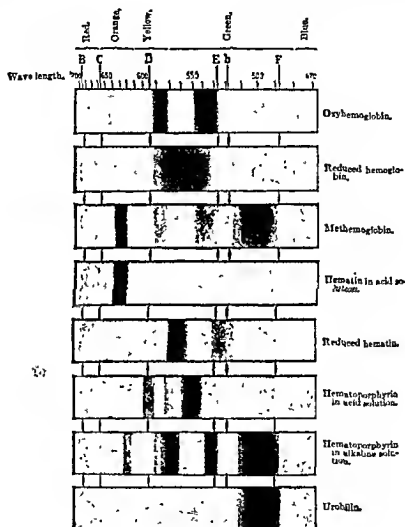

Fig. 109.—Absorption Spectra

(After Seifert and Muller.)

silver nitrate by means of thiocyanate using ferric ammonium sulphate as an indicator.

Reagents.—1. *Silver Reagent.*—Dissolve 29.012 grams silver nitrate in 150 c.c. water. Dissolve 150 grams ferric alum in 350 c.c. water with the aid of heat. Cool somewhat and transfer both solutions to a liter volumetric flask. Add 400 c.c.

concentrated nitric acid. Cool to room temperature and dilute to mark. One c.c. equals 10 milligrams sodium chloride.

2. *Ammonium Thiocyanate Solution.*—Dissolve about 14 grams ammonium thiocyanate in a liter of water. Mix thoroughly. Place in the buret. Place 10 c.c. of the silver reagent in a 100 c.c. casserole, add 20 c.c. water and titrate to the first permanent salmon color.

$$t = \text{c.c. thiocyanate titration}$$

To a 1000 c.c. volumetric flask add $1000 - 100t$ c.c. of water and add the thiocyanate solution to the mark. Mix. The solution should be exactly equal to the silver. Check by repeating the titration.

Procedure (Volhard-Harvey).—Transfer 5 c.c. of urine to 100 c.c. casserole; add 20 c.c. water, and 10 c.c. of silver reagent. Titrate with thiocyanate from the buret. The first permanent salmon-red color is the end-point.

Calculation:

$$t = \text{c.c. thiocyanate titration}$$

$$(10 - t) \times 0.01 \times \frac{\text{volume of urine excreted in c.c.}}{5} = \text{grams of sodium chloride}$$

or

$$(10 - t) \times 0.002 \times \text{volume of urine in c.c.} = \text{grams of sodium chloride excreted.}$$

Notes.—1. The sodium chloride in the urine depends on the amount eaten in the diet. It is useful in checking the efficacy of a salt-free diet.

2. The rate of chloride excretion is at times employed as a test of kidney function.

DETECTION OF MELANIN

Tests for Melanin—(a) Add a few drops of a solution of ferric chloride to 10 c.c. of urine. If melanin is present a gray precipitate forms which blackens on standing.

(b) Mix equal parts of urine and bromine water. If melanin is present a yellowish precipitate forms which gradually turns black.

Blackberg and Wanger's Test.[8]—1. Evaporate a 24-hour specimen of urine to one-third of its original volume and for each 100 c.c. of concentrated urine, add 1 gm. of potassium persulphate.

2. Allow to stand 2 hours and add an equal volume of absolute methyl alcohol; mix and allow the precipitated melanin to settle.

3. Filter off the precipitate, wash with water until the washings are colorless, and then wash with methyl alcohol to remove any remaining soluble pigments.

4. Finally, wash with ether.

5. If melanin is present a brownish-black precipitate remains on the filter. The precipitate is soluble in 5% sodium hydroxide solution from which it may again be precipitated by the addition of acid.

[8] *J. Am. M. Ass.*, 1933, 100:334.

DETECTION OF FORMALDEHYDE

Principle.—In the use of hexamethylenamine (urotropin) as a urinary antiseptic, its action depends on the liberation of formaldehyde in acid urine. To inhibit bacterial growth, 1 part of formaldehyde must be present in 5000 parts urine, a condition that is secured in only about 50% of patients who are taking this treatment.

Burnam's Test.—1. Remove any albumin by careful boiling of urine and filtration.

2. Cool about 10 c.c. of this filtered urine in a test tube to about 37° C.

3. Add 0.2 c.c. each of 0.5% phenylhydrazine hydrochloride and 5% sodium nitroprusside.

4. Run a few drops of 20% sodium hydroxide down the wall of the test tube and observe it as it diffuses through the mixture.

5. If sufficient formaldehyde is present the mixture will assume a purplish blue color that changes rapidly to dark green, light green and finally pale yellow.

6. Insufficient formaldehyde is indicated by the formation of reddish color changing to pale yellow.

MICROSCOPIC EXAMINATION

General Recommendations.—1. As far as possible specimens should be examined within six hours after voiding.

2. Unless kept at a low temperature, twenty-four-hour specimens should have a preservative added.

3. Alkaline specimens cloudy with phosphates and obscuring other elements may be slightly acidified with dilute acetic acid to redissolve them.

4. Highly acid specimens containing heavy sediments of urates obscuring other elements may be slightly warmed to redissolve them.

5. If centrifuging is not employed, the sediment should be allowed to collect by gravity (preferably in a conical container) and examined before other tests are conducted.

6. Centrifuging, however, is required for the examination of small amounts of sediment and is advised routinely.

Qualitative Method.—1. Secure sediment by centrifuging at least 15 c.c. for three to five minutes or by allowing the urine to stand at least six to twelve hours in a cool place for settling by gravity (preferably in a conical container).

2. Remove a drop of sediment by means of a pipet and place on a slide. The pipet may be a piece of tubing drawn to a blunt point and fitted with a nipple. Eight may be prepared at one time by using slides of ordinary window glass, 4 by 8 inches, divided by painted lines into 8 compartments. The stage of the microscope may be extended by a wooden table, but this is not absolutely necessary.

3. Cover glasses are not essential for ordinary examination but are advisable for high-power examinations.

4. The examination must be completed before drying takes place.

5. Examine with low power and with oblique illumination obtained by swinging the mirror a little out of the optical axis. *Too strong illumination and too great magnification are common sources of error.*

Counting Chamber Method.—A counting chamber may be used, especially for counting leukocytes (pus cells).

1. The urine should be fresh, well shaken and examined as soon as possible after collection. Do not centrifuge.

2. Fill a Thoma leukocyte pipet to the mark 1 with diluting fluid (5 per cent solution of glacial acetic acid), and to 11 with urine.

3. Shake well. Discard 2 or 3 drops. Place a drop in a Fuchs-Rosenthal chamber as used for counting cells in spinal fluid. Adjust the cover glass.

4. Wait five minutes for the cells to settle.

5. With the 16 millimeter objective and 10 × ocular, count all the cells in the entire ruling.

6. Multiply by 0.35 to give the number per c.mm.

7. Multiply by 350 to give the number per c.c. of undiluted urine.

The Addis method is described in Chapter VII.

FORMED ELEMENTS IN URINE

Unorganized Sediments

Crystals in Acid Urine.—1. *Uric Acid Crystals and Urates.*—These do not appear normally in urine at the time of voiding. All acid urines upon standing, particularly when cold, will precipitate the uric acid normally present in solution in the form of crystals or as amorphous urates. *Therefore, the finding of uric acid crystals in other than freshly voided specimens is of no clinical significance.* The urates produce cloudiness and in highly concentrated specimens may appear milky or have a pinkish or reddish color. The former may be mistaken for pus and the latter for blood. A simple test is to warm the specimen, whereupon the urates will redissolve and the specimen will become cleared of this precipitate. The crystals may settle out and appear as red grains (gravel or red sand). Microscopically they vary greatly in size and shape (Fig. 110(3) and Fig. 111(1)). The typical ones have a yellow or reddish brown color. This color is due to urinary pigment, chiefly uro-erythrine. Colorless crystals are sometimes seen. If these are hexagonal, they may resemble cystine. All colored crystals found in acid urine can safely be considered as uric acid irrespective of their shape. They are soluble in sodium hydroxide, insoluble in hydrochloric or acetic acid, and dissolve in ammonia with the formation of crystals of ammonium urate.

2. *Calcium Oxalate.*—These crystals commonly found in acid urine may occasionally be seen in neutral or slightly alkaline urine. Their presence is of clinical significance only when found immediately after voiding. The common cause of their presence is the ingestion of foods which are rich in oxalic acid such as tomatoes, spinach, rhubarb and asparagus. They usually appear as colorless, octahedral crystals, having the appearance of small squares crossed by two in-

tersecting diagonal lines, the so called "envelope crystals." They vary greatly in size and may occur as dumb-bells or spheres (Fig. 111●). They are soluble in strong hydrochloric acid, and recrystallize upon the addition of ammonia.

3. *Cystine.*—Cystine is one of the amino-acids formed in the decomposition of protein. It is present in traces in normal urine, but only when an excessive amount
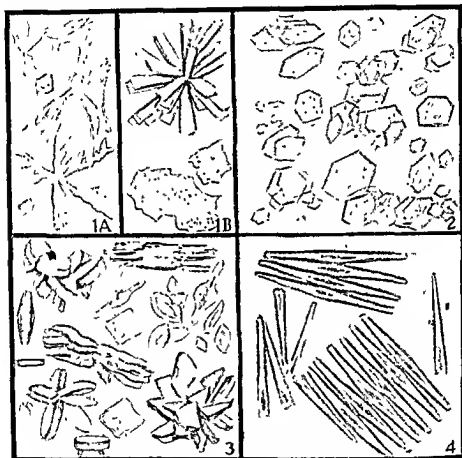

FIG. 110.—URINARY SEDIMENTS

1A, triple phosphates (feathery type); 1B, calcium phosphate; 2, cystine; 3, uric acid crystals; 4, diatoms.

is present are crystals formed. They are rarely seen. They occur as colorless, highly refractive, hexagonal plates with well defined edges, and are soluble in hydrochloric acid and insoluble in acetic acid. (See Fig. 110(2).)

4. *Leucine and Tyrosine.*—These substances rarely occur in urine. Both are cleavage products of the protein molecule and usually occur together. *Leucine* appears in the forms of yellowish, oily looking spheres, many with radial and concentric striations. It is not soluble in hydrochloric acid or in ether. *Tyrosine*

appears as very fine needles, which may appear black and are usually arranged in sheaves, with a constriction in the middle. It is soluble in ammonia and hydrochloric acid, but not in acetic acid.

Leucine and tyrosine crystals are difficult to recognize by their morphology, as other substances may take similar or identical forms, see Figure 112(4).
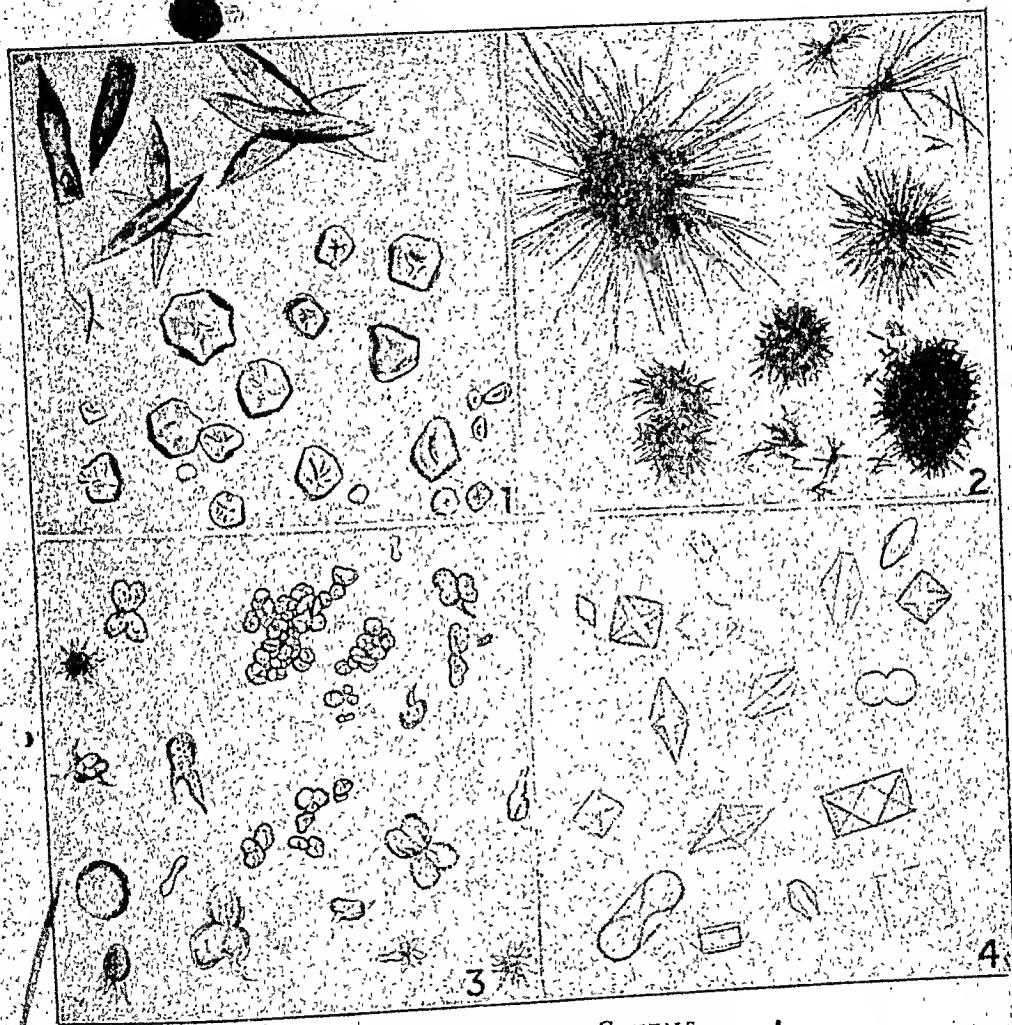
FIG. 111.—URINARY CRYSTALS

1. Uric acid (after Riedert). 2. Calcium urate (after Riedert). 3. Acid ammonium urate (after Riedert). 4. Calcium oxalate (after Todd and Sanford).

Crystals in Neutral Urine.—1. Any of the crystals found in acid or alkaline urine may occur in neutral specimens.

2. *Dicalcium phosphate* may be found in the form of colorless prisms arranged in stars or rosettes in feebly acid, neutral or feebly alkaline urines (Fig. 110(1B)).

Crystals in Alkaline Urine.—1. *Ammonio-magnesium Phosphate Crystals (Triple Phosphates).*—These crystals are commonly found in alkaline urine. The typical shape is a prism with oblique ends often called the "coffin-lid" form.

They may, when rapidly precipitated, take feathery or leaf-like forms (see Fig. 110(1A) and Fig. 112(2)).

2. *Amorphous Phosphates.*—Common in alkaline urine and appears as a granular precipitate (see Fig. 112(1)).

3. *Acid Ammonium Biurate.*—It is precipitated only when free ammonia is present and is therefore usually found along with phosphates in decomposing urine.
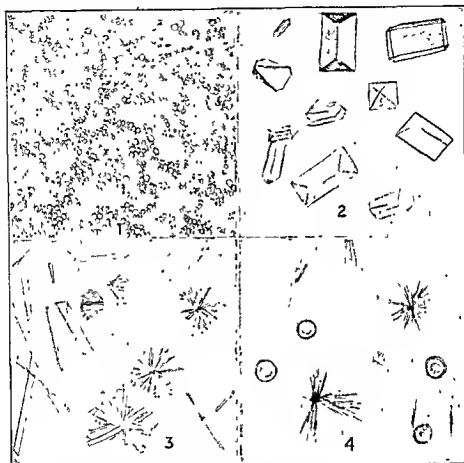
FIG. 112.—URINARY CRYSTALS

1. Amorphous phosphates (after Riedert). 2. Triple phosphates (after Todd and Sanford).
3. Calcium sulphate (after Riedert). 4. Leucine and tyrosine (after Riedert).

It occurs in the form of yellow spheres with a spicule, the so called "thorn-apple crystals." Occasionally they form sheaves of fine needles, and rhizome forms (see Fig. 111(3)).

4. *Calcium Carbonate.*—Occurs as amorphous granules or colorless spheres and dumb-bells. It dissolves readily in acetic acid with the formation of gas.

All of the precipitates found in alkaline urine are soluble in acetic acid.

ORGANIZED SEDIMENTS

Epithelial Cells.—The authors agree with Kilduffe's statement[9] "that the accurate classification of the epithelial cells seen in urine requires great skill; that in many cases it is impossible, and that in the hands of the ordinary observer, deductions based upon their presence are apt to be erroneous."
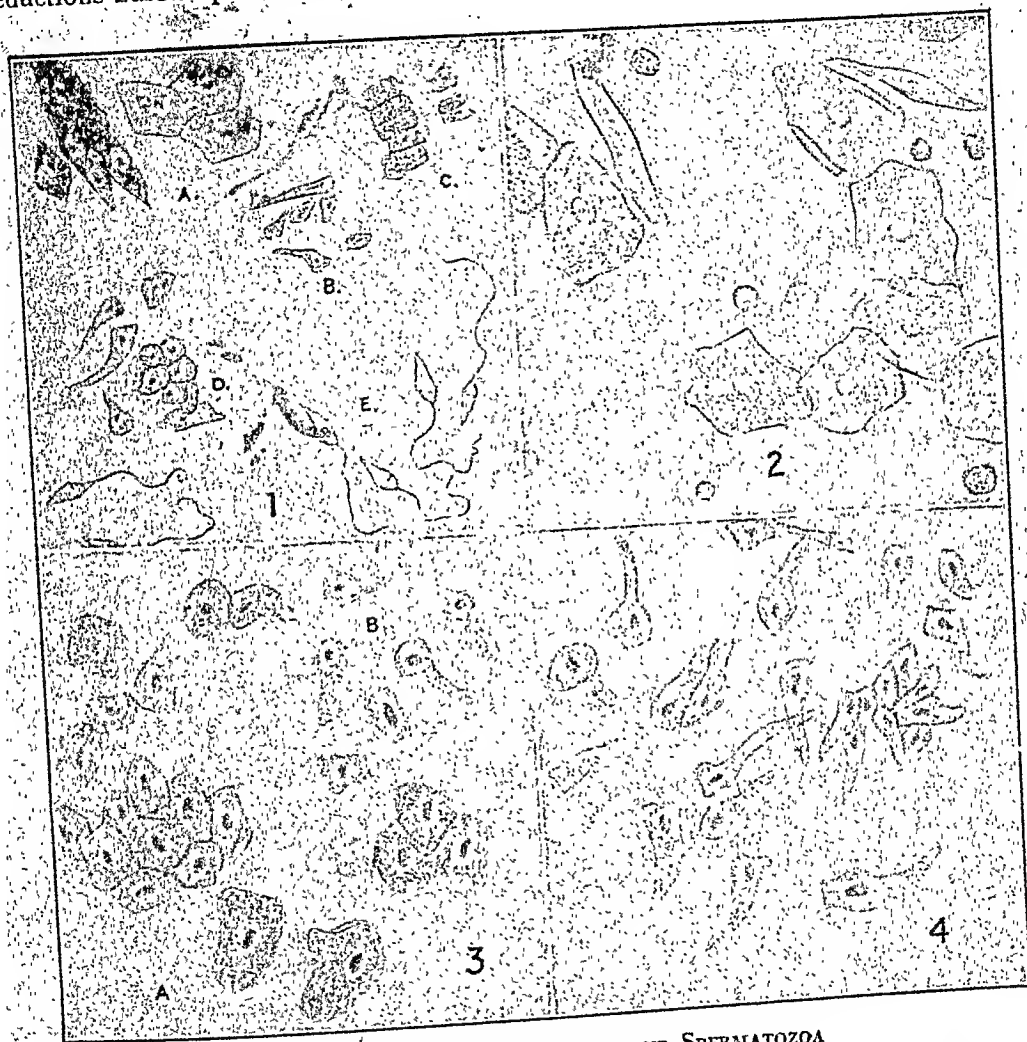
FIG. 113.—URINARY EPITHELIUM AND SPERMATOZOA

1. *A*, Vaginal epithelium; *B*, ureteral epithelium; *C*, renal epithelium; *D*, epithelium from pelvis of kidney; *E*, spermatozoa (all after Riedert). 2. Squamous epithelium and pus cells (after Todd and Sanford). 3. Epithelium from urethra (*B*) and bladder (*A*) (after Todd and Sanford). 4. Epithelium from pelvis of kidney (after Todd and Sanford).

1. *Squamous or Pavement.*—These are large flat cells with a small distinct round or oval nucleus (Fig. 113(1)). They come from ureters, bladder or the genitalia; as a rule, except in specimens from the female, they are present in rela-

9 R. A. Kilduffe, "Urinalysis," *The Cyclopedia of Medicine*, Vol. XII, 570, F. A. Davis Co., Philadelphia, 1934.

tively small numbers. Specimens from the female are commonly contaminated with cells of this type originating from the vagina. Although the vaginal cells are especially large, thin, angular and frequently rolled up, it is very difficult to determine the source of these cells in such cases with any degree of certainty.

2. *Small Round or Polyhedral.*—These cells are somewhat larger than the
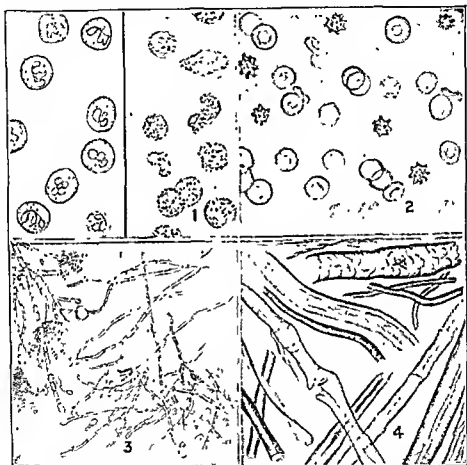
FIG. 114.—URINARY LEUKOCYTES, ERYTHROCYTES, MOLDS AND ARTEFACTS

1. Leukocytes (after Todd and Sanford). 2. Erythrocytes (after Todd and Sanford). 3. Molds (after Riedert). 4. Artefacts (after Riedert).

polymorphonuclear leukocyte and have a single round nucleus. They come from the uriniferous tubules or the deeper layers of any part of the urinary tract. Only when these cells are found adhering to casts can they be considered as of renal origin. See Figure 113.

3. *Caudate.*—These are smaller than the squamous cell and have tail-like processes. They come most frequently from the pelvis of the kidney but may also originate from the neck of the bladder. See Figure 113.

Leukocytes.—A small number of leukocytes are present in normal urine. Any marked increase in their number is significant of disease somewhere along the urinary tract in males, and from either the urinary or genital tracts in the female, unless the specimen is obtained by catheterization. Contamination of the specimen with vaginal discharge may introduce large numbers of leukocytes. Dead leukocytes are called *pus cells* (Fig. 114). There is no way of determining whether a leukocyte is dead or alive except in the case of those showing degenerative changes such as swelling. disintegration and a tendency to aggregate in clumps. It is common practice to call them leukocytes when they occur in normal numbers and *pus*
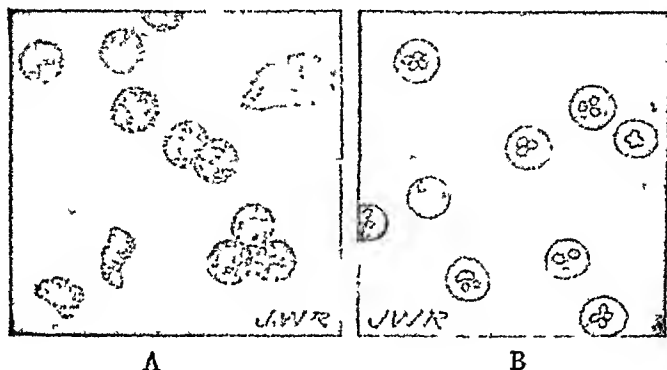
FIG. 115.—PUS CORPUSCLES IN URINE

A, as ordinarily seen. At the lower left are two ameboid corpuscles. The large structure at the right is a bit of degenerated epithelium; *B*, when treated with acetic acid (×475). (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia).

cells when they are definitely increased. The nuclei are frequently indistinct or obscured by granules. The addition of a little dilute acetic acid brings the nuclei clearly into view. See Figure 115.

Pus when at all abundant adds an appreciable amount of albumin and forms a white sediment resembling that produced by phosphates. The leukocytes usually found in urine are neutrophils (polymorphonuclear). Occasionally plasma cells are present, usually with neutrophils. These have a single round nucleus, located at one side of the cell. In alkaline urines the pus may be transformed by the alkalis into a gelatinous substance giving the urine a mucilaginous consistency.

Erythrocytes.—These cells are not found in normal urine. Their presence is always pathological when contamination with menstrual discharge can be excluded. The cells may have a normal appearance or may be crenated, swollen or hemolyzed. When blood is present in a large amount, it will change the color of the urine to a hazy reddish or brown color, commonly called "smoky."

When the cells are atypical or there is any doubt, add a little dilute acetic acid. If they are erythrocytes, they will dissolve and disappear. Occult blood tests can be used as confirming tests. However, they are not sensitive enough in cases where only a very few cells are found microscopically. See Figure 114 (2).

Casts.—1. *Hyaline.*—These casts are colorless, homogeneous, semi-transparent and cylindrical in shape (Fig. 116). They are difficult to see unless the proper illumination is used. Although described as homogeneous, upon careful examination a small amount of granular material is usually seen adhering to them. They are
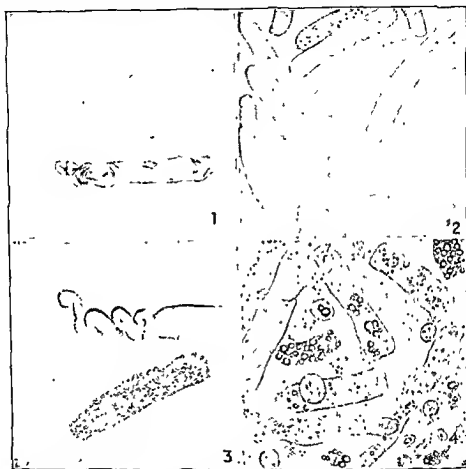

FIG. 116.—URINARY CASTS

1. Hyaline casts (after Riedert). 2. Hyaline and finely granular casts (after Todd and Sanford). 3. Waxy (colloid) and granular casts (after Riedert). 4. Granular and fatty casts (after Riedert).

usually straight with parallel sides and rounded ends. Occasionally, curved and convoluted forms are seen. They are readily soluble in acetic acid in contrast to fatty casts which are insoluble.

2. *Granular.*—These are hyaline casts containing many fine or coarse granules. Those having very fine granules are called *"finely granular"* and those having coarse granules, *"coarsely granular."* See Figure 116.

3. *Waxy.*—These are homogeneous like the hyaline cast, but more opaque. They have a dull waxy appearance and are often grayish. All gradations between

hyaline and waxy casts may be found. No doubt the more refractive hyaline casts are often incorrectly reported as waxy. See Figure 116.

4. *Fibrinous.*—These are similar to waxy casts except for their color, which is distinctly yellowish. The color is probably due to altered blood pigment.
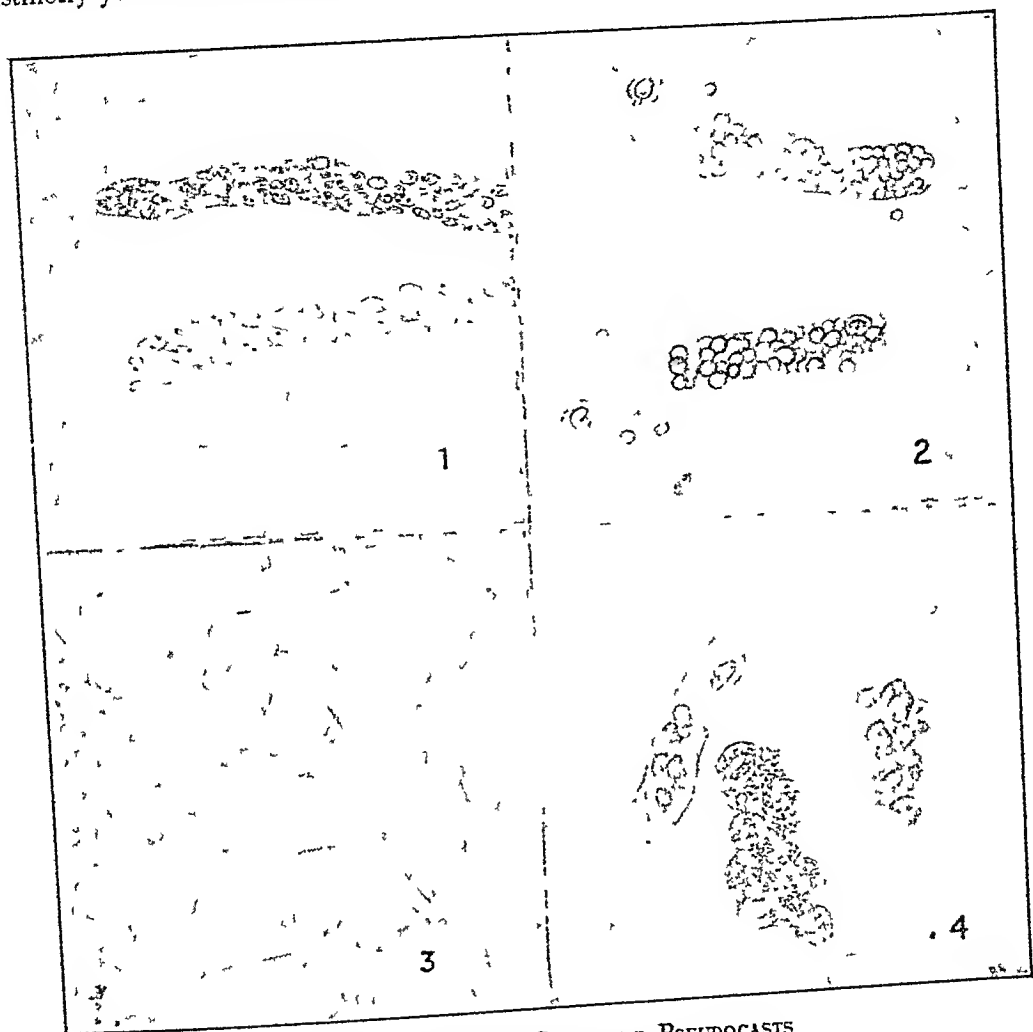
Fig. 117.—Urinary Casts and Pseudocasts

1. Epithelial casts (after Riedert). 2. Blood casts (after Todd and Sanford). 3. Mucous threads and cylindroids (after Todd and Sanford). 4. Pseudocasts composed of swollen epithelial cells (after Riedert).

5. *Fatty.*—These are casts of any kind which contain numerous fat droplets. The fatty nature of the globules can be easily determined by staining with osmic acid or sudan III. They are insoluble in acetic acid. The source of these fat droplets is chiefly from degenerated epithelial cells. See Figure 116.

6. *Epithelial.*—These contain epithelial cells from the renal tubules. Dilute acetic acid brings out the nuclei more distinctly and aids in the recognition of the cells. These casts are rare. See Figure 117.

TABLE II

TABLE OF USUAL URINARY FINDINGS IN RENAL AND URINARY TRACT DISEASE *

Condition	Amount c c	Appearance	Reaction	Sp Gravity	Albumin	Sediment
Active hyperemia	800–1200	High color	Acid	1 016–1 030	Trace	Hyaline and fine granular casts, blood cells, renal cells
Passive Congestion	400–1200	High color	Acid	1 025–1 030	Trace	Occasional hyaline casts; no blood, occasional renal cells
Acute, diffuse nephritis; acute stage	200–400	Dark, smoky	Acid	1 015–1 030	Heavy; 200–2000, plus	Heavy, hyaline, granular and blood casts; red and white blood cells, epithelial casts and renal epithelium
Second— Fatty phase	800–1500	Dark, smoky	Acid	1 011–1 025	Present; less than acute	Similar to acute state but renal cells show fatty degeneration, pus may be present.
Convalescent stage	1500–2000	Pale	Acid	1 008–1 010	Traces	Various kinds of casts in moderate numbers moderate numbers of blood and pus cells.
Chronic, diffuse nephritis	2000	Pale	Acid	1 010–1 013	Trace to 300 mg.	Numerous hyaline and fine granular casts, occasional fatty casts
Chronic Parenchymatous nephritis	Normal to decreased	Normal to high	Acid	1 020–1 025	Large amount 300–2000 mg.	Heavy; all kinds of casts; red blood cells; fatty epithelium.
Chronic Interstitial nephritis, 1st stage	1200–2000	Normal	Acid	1 012–1 018	Trace	More casts than first stage with granular epithelium.
Advanced stage	2000–3000	Pale	Faint, acid	1 010–1 015	100 to 250 mg.	Numerous hyaline and granular casts. Occasional fatty cast.
Late stage	1500	Watery	Faint, acid	1 005–1 010	Trace to 100 mg.+	Very few hyalin and granular casts.

Senile interstitial nephritis	1500	Normal or high	acid	1.020-1.025	Trace	Occasional hyaline and granular cast; renal cells.
Pyelitis: Acute	100-1000	Reddish (hematuria)	Acid	1.025-1.030	100-500 mg.	Pus, blood, and occasional granular cast.
Chronic	1200	Pale	Acid	1.010-1.015	Truce	Pus, small round cells, occasional cast.
Prostatitis: Acute	500-1000	Bloody	Acid	1.025-1.030	Heavy: 500 mg.	Blood, pus, spermatozoa, renal and prostatic epithelium.
Chronic	800-1200	Pale	Acid or alkaline	1.015	Trace to 500 mg	Similar to acute, but less blood.
Cystitis	Normal	Very turbid	Acid or alkaline	1.015-1.025	Trace to 200 mg.	Abundant pus cells; much mucus and bacteria.
Urethritis	800-1200	Turbid, second specimen clear	Acid	1.020-1.025	Trace or absent	Much pus and urethral epithelium; phagocytized cort.
Amyloid kidney	2000-1000	Pale (greenish)	Acid	1.012-1.018	Trace	Hyaline, granular and waxy casts. No blood.
Diabetes mellitus	3000-6000	Watery	Faint, acid	1.025-1.010	Absent; sugar present	Occasional hyaline and granular casts.
Diabetes insipidus	5000 plus	Watery	Acid or neutral	1.001-1.005	Absent (no sugar)	Leukocytes and squamous epithelium.

* R. A. Kilduffe, "Urinalysis," The Cyclopedia of Medicine, Vol. XII, 570, F. A. Davis Co., Philadelphia, 1934.

7. **Pus.**—These are composed almost entirely of pus cells. Finding an occasional pus cell in casts has no special significance. It is only when the cast is nearly or completely filled with pus cells that they are reported as pus casts.

8. **Blood.**—These contain many erythrocytes which are often degenerated. See Figure 117.

Pseudocasts.—1. *Mucous threads* often appear as long strands resembling hyaline casts. They are more ribbon-like, tapering at the ends and have less defined edges. See Figure 117.

2. *Urates or phosphates* may aggregate into cylindrical masses resembling granular casts. The application of heat will dissolve the urates, and acetic acid the phosphates.

Cylindroids.—These are formations which closely resemble hyaline casts. They are, however, longer, more ribbon- or band-like and taper to a slender tail which is often twisted or curled. See Figure 117.

Spermatozoa.—These are usually present in specimens collected after nocturnal emissions or prostatic massage and as the result of vaginal contamination after coitus. They are easily recognized. See Figure 113.

Bacteria.—Normal urine does not contain bacteria. However, urine is readily contaminated when passing through the urethra and with vaginal secretions in the female. Since urine is a very good culture medium for many bacteria, multiplication takes place rapidly. Bacteria when in large numbers, cause uniform cloudiness of the specimen which will not clear by filtration. In disease, pathogenic bacteria may be present, which require special examination for their detection.

Extraneous Matter.—The accidental contamination of urine with extraneous matter is not at all uncommon. The source is usually the use of unclean containers or utensils for collecting specimens. *Yeasts* are often found in specimens which have stood for some time. They multiply rapidly, particularly in urines containing sugar. They may be mistaken for erythrocytes from which they may be distinguished by their oval shape, tendency to form chains, the presence of budding forms and their insolubility in acids or alkalis. *Molds* are commonly found in urine which has stood 24 hours or more (Fig. 114(3)). *Fibers of wool, cotton, linen or silk* may come from clothing, towels or the air (Fig. 114(4)). *Oil droplets*, which are highly refractive, may come from oiled catheters or unclean containers. *Diatoms* from tap water are not uncommon (Fig. 110(4)).

CHAPTER VI

METHODS FOR THE HORMONAL DIAGNOSIS OF EARLY PREGNANCY, HYDATIDIFORM MOLE, CHORION-EPITHELIOMA AND TERATOMATA OF THE TESTICLE

By Israel Davidsohn and Harry L. Reinhart

Principles.—During pregnancy there is an increase in the amount of gonad stimulating factors (anterior pituitary hormone?) excreted in the urine. In the presence of hydatidiform mole and of chorionepithelioma the hormone is present in the urine in much larger quantities than in pregnancy. In the presence of a teratoma of the testicle there is also a marked increase of the hormone. In normal man and woman there are from five to ten mouse units per liter of urine. When urine containing an increased amount of this hormone is injected into female animals, such as mice, rats or rabbits, it produces marked changes in the ovaries. The changes are not observed in animals injected with urine from non-pregnant women. The tests are stated to be reliable after the tenth day following the first missed menstrual period following suspected conception. They remain positive until 7 days after the birth of a full term baby and for 10 to 16 days after an abortion in the early months of pregnancy.

THE ASCHHEIM-ZONDEK TEST [1]

1. Collect morning specimen of urine in clean container. (Sterile precautions are not necessary.) If specimen is to be sent by mail or in case of delay for other reasons, preserve by adding about 4 grains of boric acid crystals per 100 c.c. of urine. In the laboratory, keep the specimen on ice.

2. Adjust reaction to slightly acid if specimen is alkaline.

3. Filter through paper or centrifuge and use supernatant urine.

4. Mice or rats can be employed. If mice are used they should be young females weighing between 8 and 10 grams (21 days of age). Inject 5 animals under the skin of the back with 6 doses of 0.5 c.c. of urine distributed over 2 days.

5. If rats are used they should be young females, thirty to forty-five days old and not weighing over 65 grams. Only one rat is used for each test. Inject subcutaneously with 0.5 c.c. of urine twice daily on three successive days.

6. Ninety-six hours following the first injection kill the animal with an anesthetic or with illuminating gas and examine the genital organs. Positive results have been observed as early as sixty hours after the first injection. If speed is indi-

[1] S. Aschheim, "Pregnancy Test," *J. Am. M. Ass.*, April, 1935, 104:1324-29.

cated (as in suspected ectopic pregnancy) use a large number of mice, examine a few after sixty hours, and if the result is negative examine the other mice after ninety-six hours.

7. In positive cases the ovaries are enlarged, hyperemic and show hemorrhagic spots and yellowish protrusions which are due to hemorrhages into the follicles and corpora lutea respectively. The uterine horns are enlarged, the hymen open and smears of the vaginal secretion show changes in the cell types due to estrum. These vaginal changes are due to female sex hormone and therefore alone cannot be considered as positive findings. Histological examination of sections of the ovaries may be helpful.

THE FRIEDMAN TEST [2]

1. Collect urine as described under Aschheim-Zondek test.

2. Filter.

3. Inject two doses of 10 c.c. each at about six hours' intervals in the ear vein of a female rabbit which should be not less than seventeen weeks old and should weigh not less than 1500 grams. One dose of 13-15 c.c. is probably preferred. Sterile precautions are not necessary, but the urine should be warmed up to room temperature before the injection. The rabbits should be obtained from a reliable breeder. They should be separated at weaning and kept in individual cages for three to four weeks before use. Some authors [3] prefer mature nonpregnant rabbits not less than six months old and weighing not less than 2.5 kilograms. They should have had a litter and been isolated for three to four weeks preceding the test. Such mature rabbits should receive two injections of 15 c.c. of urine.

4. Anesthetize the rabbit forty-eight hours after first injection and examine ovaries. Ether or intravenous injection of sodium amytal (60 mg. per kg. of body weight in distilled water) 30 to 40 minutes before operation are recommended as anesthetics or local using 2% novacain. Place rabbit on operating board and elevate the caudal end of the animal to permit a better view of the genital organs.

5. If negative, the ovaries remain small in size and show no change (see A in Plate IV) ; if positive, from 1 to 14 corpora haemorrhagica and corpora lutea are found present in each ovary. In some instances a positive result has been obtained in twelve hours and Schneider [4] recommends injecting two rabbits in cases in which a diagnosis of pregnancy might influence a decision regarding operation. The first rabbit is then examined at twelve to twenty-four hours and the result checked by examination on the second rabbit at forty-eight hours without loss of time. If one ovary appears negative the other must be examined, but if one ovary)

[2] M. H. Friedman and M. E. Lapham, "Simple, Rapid Procedure for Laboratory Diagnosis of Early Pregnancies," Am. J. Obst. & Gynec., March, 1931, 21:405-410.

[3] H. L. Reinhart, "The Results of Two Years Experience with the Friedman Test," Am. J. Clin. Path., Jan., 1933, 3.915.

L. Davy and E. L. Sevringhaus, "Analysis of Errors Inherent in Pregnancy Tests Based on Aschheim-Zondek Reaction," Am. J. Obst. & Gynec., Dec., 1934, 28:888-901.

[4] P. F. Schneider, "A Hormone Test for the Diagnosis of Early Pregnancy," Surg. Gynec. & Obst., 1931, 52:56-60.

appears positive the other need not be inspected. It is well to remember that some rabbits, though fortunately only few, are refractory to the injection of gonad stimulating factors and fail to show the characteristic changes in the ovaries even when injected with the urine of known pregnant patients. This seems to be more often the case in young than in older rabbits. Such rabbits have commonly small and poorly developed ovaries. Therefore, if the ovaries appear small and the follicles indistinct, it is advisable to inject the rabbit with 10 c.c. of urine from a known pregnant patient, and to withhold the final report until the rabbit was shown to react to a known positive urine. If it remains negative then a new rabbit has to be injected.

Negative rabbits can be used again immediately. Rabbits with positive results can be used after an isolation for ten to fourteen days. The abdominal wound should be closed in two layers.

6. Toxicity of the urine for animals have been occasionally found due to drugs taken by the patients. Different detoxifying methods have been recommended.

Detoxifying Method of Zondek.—Filter 30 c.c. of fresh urine and add 90 c.c. of ether. Shake vigorously in a separatory funnel for five minutes and allow to layer. The urine layer is drawn off into an open beaker and allowed to stand in the open air until the residual ether evaporates. Dissolve 0.9 gm. of glucose in this ether-freed urine, and keep the specimen in a refrigerator until used.

Good results were obtained by washing the urine three times with equal quantities of ether, discarding the latter and letting all traces of it evaporate from the urine.

QUANTITATIVE ASCHHEIM-ZONDEK TEST FOR THE DIAGNOSIS OF HYDATIDIFORM MOLE AND CHORIONEPITHELIOMA [5]

Inject groups of 5 mice with 6 doses of 0.5 c.c. each of urine diluted 1:10, 1:50, 1:100, and 1:1000. The technic is the same as for the qualitative Aschheim-Zondek test. If the dilution of 1:10 gives a positive result then no less than 3.330 mouse units of hormone are assumed to be present per liter of urine, with corresponding higher values if the higher dilutions give positive results. A negative test does not exclude chorionepithelioma.

QUANTITATIVE ASCHHEIM-ZONDEK TEST FOR THE DIAGNOSIS OF TERATOMA OF TESTIS [6]

In about one-third of these tumors the amount of hormone eliminated in the urine is less than 2,000 mouse units per liter. To recognize these cases the urine must be concentrated according to the method of Ferguson.

Preparation of Concentrate.—1. The fresh morning specimen of urine is filtered if cloudy, and if alkaline is rendered faintly acid to litmus paper with a few drops of weak acetic acid.

2. Add 100 c.c. of 95% alcohol to 20 c.c. of this urine in a graduate and mix

[5] M. L. Leventhal and W. Saphir, *J. Am. M. Ass.*, Sept., 1934, 103:668.
[6] R. S. Ferguson, *Am. J. Cancer*, 1933, 18:269-295.

by inverting several times. The mixture is allowed to stand overnight, the hormone separating out in the precipitate.

3. The following morning the supernatant fluid is siphoned off, leaving about 20 c.c. of fluid over the precipitate. This is centrifuged for 5 minutes at 2000 r.p.m. The supernatant fluid is poured off.

4. Add 30 c.c. of ether to the precipitate and mix by stirring with a glass rod for ten minutes. Again centrifuge for five minutes and pour off the ether.

5. Distribute the precipitate around the bottom of the tube, using a glass rod, and allow it to dry. Add 4 c.c. of distilled water; mix, and allow to stand over-night.

6. Centrifuge this mixture the following morning. The water now contains the hormone; it is pipetted off and kept in the refrigerator until used. This extract is a 5x concentrate of the fresh urine. Stronger extracts may be made by modifying the method.

Six mice are used. Three are given 5 doses of 0.1, 0.2, and 0.4 c.c. of fresh urine and the other three similar doses of the concentrate.

According to Ferguson, there is a direct relation between the embryonal character of the tumor and the quantity of the excreted hormone. The highest amounts are found in chorionepithelioma, less in the embryonal adenocarcinoma and least in the teratoma of adult type. It disappears after successful removal of the tumor, decreases in quantity after irradiation, increases in recurrences and in the presence of metastases. However, further studies are needed to confirm those views and it must be remembered that a negative hormone test does not exclude malignancy of the testis.

CHAPTER VII

METHODS FOR CONDUCTING TESTS OF KIDNEY FUNCTION

Principles.—1. In approaching the subject of renal functional diagnosis one must bear in mind the fact that tests of kidney function are designed to indicate only the functional efficiency of the kidneys under the conditions existing at the time and with regard to the particular function or functions to which the test applies. The chief source of disappointment in the use of these methods comes from attempts to interpret the results obtained in terms of the probable nature and extent of renal disease.

2. It must be realized that functional diagnosis and disease diagnosis are by no means synonymous terms and that marked functional impairment may exist in the absence of extensive demonstrable tissue damage, and vice versa. The ability of the kidneys to function efficiently may be affected by certain primarily extrarenal factors, including profound circulatory disturbances (shock, congestive heart failure), obstructive urinary tract lesions and prerenal deviation of water (vomiting, diarrhea and edema), and, conversely, because of their wide margin of safety, they may be able to function efficiently despite the existence of extensive organic disease.

3. Furthermore, the prognostic significance of abnormal functional findings is dependent upon the nature of the underlying pathologic process. For example, marked functional impairment in chronic glomerulonephritis is usually of ominous portent, whereas a similar degree of impairment in acute glomerulonephritis is not necessarily so, kidney function returning to normal if the renal lesion heals completely, as it so often does.

4. A sharp distinction must be made, therefore, between methods of functional diagnosis and disease diagnosis. The value of the former must be obvious, particularly in view of the fact that other clinical methods and the evaluation of subjective manifestations are notoriously unreliable in estimating the presence or extent of renal functional impairment.

CONCENTRATION TESTS FOR RENAL FUNCTION

Principles.—Broadly speaking, one of the most important functions of the kidney is to eliminate a certain required quantity of solids, organic and inorganic. in a certain amount of water, which varies normally according to the intake and to its elimination through other channels. In the process of their removal from the blood and their passage through the uriniferous tubules the concentration of these solids is tremendously increased and a saving of water is effected which is

essential to the maintenance of normal body functions. By virtue of this ability to excrete solids in high concentration, the normal kidney can eliminate the required amount, within wide limits, regardless of the amount of water available for their solution. Increasing impairment of kidney function is reflected in and, indeed, consists in a progressive decrease in concentrating ability. Concentration tests of kidney function are designed to reveal the maximum urinary concentration that can be obtained under standardized conditions, usually consisting of a normal solid and a limited fluid intake.

The procedure advocated by Fishberg is most satisfactory because of its simplicity. The methods of Volhard and Fahr and of Lashmet and Newburgh are also extensively employed.

Method of Fishberg.—1. High protein, low fluid (200 c.c.) supper the evening before (6 to 7 P.M.)

2. Allow no fluids after this until the test is completed.

3. Discard all urine passed during the night.

4. On awakening, empty the bladder and save as specimen No. 1.

5. Stay in bed and empty bladder one hour later. Label specimen No. 2.

6. The patient may now get out of bed and void again in one hour. This is labeled specimen No. 3.

7. The specific gravity of each specimen is determined.

8. *Normally* the specific gravity of at least one sample should be 1.022 or higher.

Method of Volhard and Fahr.—1. Allow no fluids from the evening before the test until the test is finished, and no food between meals.

2. 8 A.M., breakfast: Dry cereal with sugar, syrup, or honey; no milk; one egg; toast or bread with butter.

3. 12 noon, dinner: Roast beef, steak, or chops; potatoes, boiled, baked or riced; bread and butter; jam.

4. 5 P.M., supper: Two eggs; bread and butter; jam.

5. 8 A.M. of same day: Empty bladder. Collect urine in separate containers every three hours thereafter until night, that is, at 11 A.M., 2 P.M., 5 P.M., 8 P.M.; and collect all urine from 8 P.M. to 8 A.M. next morning in one container.

6. Note the quantity and specific gravity of each three-hour sample and of the twelve-hour sample and plot as a curve.

7. *Normally* the specific gravity of at least one specimen should be 1.025 or higher.

Method of Lashmet and Newburgh.—1. At 10 P.M. of the night preceding the test withhold all food and fluid, except the special diet, for the succeeding 38 hours. This diet consists of: protein, 40 gm.; fat, 10.4 gm.; carbohydrate, 20.4 gm.; 1900 calories; 9.1 gm. of inorganic solids and an excess of base equivalent to 18 c.c. of normal solution. One gram of NaCl is added to the daily diet and no fluid as such is allowed.

2. At 8 A.M. of the test day, during which only the above diet is administered, empty bladder and discard urine.

3. Collect all urine from 8 A.M. to 8 P.M. as specimen 1.

4. At 10 A.M. following day collect urine as specimen 2.

5. At 12 noon collect urine as specimen 3.

6. Determine specific gravity of the three specimens.

7. *Normally* the specific gravity of the urine should be at least 1.026.

Significance of Concentration Tests.—1. In all concentration tests. if the urine contains significant amounts of protein, correction should be made for the effect of the latter by subtracting the factor. $0.003 \times$ grams protein per 100 c.c., from the observed specific gravity.

2. Impairment of the ability of the kidneys to eliminate a concentrated urine is one of the first detectable evidences of impaired renal function. The greater the functional damage, as a rule. the lower the concentrating ability and the maximum urinary specific gravity becomes progressively lower regardless of the urinary volume, approaching 1.010, at which point the urine is approximately isotonic with *deproteinized blood plasma*.

3. Impaired concentrating ability may be masked in diabetes mellitus due to the effect of the urinary sugar in increasing the specific gravity. Contrariwise, subnormal values may be obtained in the presence of normally functioning kidneys during periods of elimination of edema fluid and in diabetes insipidus.

4. In the absence of glycosuria. normal findings with the concentration test may be regarded as evidence of normal renal function.

5. The degree of functional impairment is not usually indicated as exactly as by the urea clearance test. However, in recovering cases of acute glomerulonephritis, persistently low maximum specific gravities may indicate residual damage for some time after the blood urea clearance has returned to normal.

TEST MEAL FOR RENAL FUNCTION

Principle.—Under standardized conditions of food and fluid intake the urinary specific gravity normally varies inversely as the volume and fluctuates within a certain minimum range when samples are examined at frequent intervals during the day. The night urine is usually of lower volume and higher specific gravity than the day urine. When renal function is impaired the flexibility of renal excretion in response to the varying demands during the 24-hour period is diminished, the difference between day and night urine becomes less marked and the extent of the variation in specific gravity of individual specimens diminishing, the specific gravity tending to become more fixed at a relatively low level regardless of the volume of the specimens.

Method of Mosenthal.—1. Upon the day of the test, and preferably also the day before, place the patient upon a full diet, such as the following: breakfast of fruit, cereal. bread, butter and tea, coffee, cocoa or water. at 8 A.M.; dinner of soup, meat, vegetables, bread, butter, dessert and tea. coffee or water at noon; and supper of eggs, bread, butter, fruit and tea or water at 5 P.M. Much latitude is allowable in choice of foods, and in many cases the ordinary diet to which the patient is accustomed may be used. At least a pint of fluid—tea, coffee, water, etc.—

must be taken at each meal; and no food or liquid of any sort may be taken between meals until after 8 o'clock the following morning.

2. Instruct the patient to empty his bladder immediately before breakfast. Collect specimens of urine at 10 A.M., noon, 2 P.M., 4 P.M., 6 P.M., 8 P.M., and finally at 8 o'clock the following morning. It is essential that the intervals be exact and that the bladder be completely emptied each time. Should the hour for meals be changed, the times of collecting the sample of urine should be changed accordingly. The last of the two-hour specimens must not be collected less than three hours after the beginning of the evening meal.

3. Measure the night urine (8 P.M. to 8 A.M.) and take its specific gravity with an urinometer.

4. Measure the two-hour specimens and take their specific gravity, first making sure that they are all at the same temperature, since misleading figures may be obtained if some have been kept on ice and some at room temperature.

5. *Normally* the results are as follows:

(a) The night urine will be much less than the total day urine. It is usually 250 to 350 c.c., and will seldom exceed 400 to 500 c.c.; 750 c.c. is the maximum. Its specific gravity will usually be 1.018 or above.

(b) The highest specific gravity recorded for the two-hour day specimens will exceed 1.018 while the difference between the highest and lowest will be not less than 8 or 9 points. If, for example, the most concentrated specimen has a specific gravity of 1.020, the most dilute will be 1.011 or less.

Significance of the Test.—(a) Nocturnal polyuria. The volume of the night urine exceeds 750 c.c. This is usually one of the first and most definite evidences of impaired kidney function. A volume between 500 and 750 c.c. is suspicious, and usually indicates impairment.

(b) Low maximal specific gravity of day or night urine, the highest falling below 1.018.

(c) Fixation of specific gravity, that is, lessened variation in the specific gravities of the two-hour specimens. This is a very important sign of renal functional impairment. In advanced cases the difference between the highest and lowest specific gravities may be only one or two points. As a rule, the level at which the specific gravity is fixed approaches 1.011 as the functional impairment increases and the kidneys lose their ability to concentrate.

The same factors invalidating the concentration test invalidate the Mosenthal test. Occult edema must be carefully excluded before an abnormal result is attributed to renal disease. It is not as sensitive as the concentration tests and adds no information to that obtained by the methods previously described.

UREA CLEARANCE TEST

Principle.—The blood urea clearance is an expression of the number of cubic centimeters of blood that would contain, at the time of study, the quantity of urea removed from the blood by the kidneys in one minute and excreted in the urine during that time. This is usually expressed in terms of percentage of the average

normal, which varies with the body surface area and with the minute volume of urine.

Procedure.—1. The patient is allowed to eat an ordinary breakfast and is given an additional glass of water.

2. The bladder is immediately emptied completely, the specimen being discarded. The exact time is recorded at which the subject finishes voiding, which marks the beginning of Period 1.

3. At the end of approximately one hour the bladder is again completely emptied and the time noted exactly. This marks the end of Period 1. The volume of the entire specimen is carefully measured and it is preserved by storing in the refrigerator.

4. The subject is given another glass of water and blood is removed for urea N determination.

5. At the end of approximately two hours from the beginning of Period 1 the bladder is again completely emptied and the time noted exactly as marking the end of Period 2. The volume of urine is carefully measured and it is preserved in the refrigerator unless examined immediately.

6. Calculate the volume of urine excreted per minute during each period.

7. Determine the urea N concentration of each urine specimen and of the blood sample.

Calculation.—1. If the minute volume of urine exceeds 2 c.c. the expression is that of "Maximum Clearance," the formula being:

$$C_m = \frac{UV}{B} = \text{normally 64-99 c.c. (average 75 c.c.)}$$

Since the clearance is commonly expressed as the percentage of the average normal, the calculation may be simplified by using the formula,

$$\text{Percentage of average normal } C_m = \frac{100\,UV}{75\,B} \text{ or } \frac{1.33\,UV}{B}$$

2. If the minute volume of urine is less than 2 c.c. the expression is that of "Standard Clearance," the formula being:

$$C_s = \frac{U\sqrt{V}}{B} = \text{normally 40-68 c.c. (average 54 c.c.)}$$

To facilitate its expression as percentage of the average normal, as above, the following formula may be used:

$$\text{Percentage of average normal } C_s = \frac{100\,U\sqrt{V}}{54\,B} \text{ or } \frac{1.85\,U\sqrt{V}}{B}$$

U = Urea N concentration of urine (in mg. per 100 c.c.)
B = Urea N concentration of blood (in mg. per 100 c.c.)
V = Urine volume per minute

Notes.—According to Van Slyke, Page, Hiller and Kirk (*J. Clin. Invest.*, 1935, 14:901). results are somewhat more consistent if, in the formulæ given above, one uses for "U" the urinary concentration of urea N plus ammonia N instead of only urea N. The technical procedure is then also simplified since the process

must be taken at each meal; and no food or liquid of any sort may be taken between meals until after 8 o'clock the following morning.

2. Instruct the patient to empty his bladder immediately before breakfast. Collect specimens of urine at 10 A.M., noon, 2 P.M., 4 P.M., 6 P.M., 8 P.M., and finally at 8 o'clock the following morning. It is essential that the intervals be exact and that the bladder be completely emptied each time. Should the hour for meals be changed, the times of collecting the sample of urine should be changed accordingly. The last of the two-hour specimens must not be collected less than three hours after the beginning of the evening meal.

3. Measure the night urine (8 P.M. to 8 A.M.) and take its specific gravity with an urinometer.

4. Measure the two-hour specimens and take their specific gravity, first making sure that they are all at the same temperature, since misleading figures may be obtained if some have been kept on ice and some at room temperature.

5. *Normally* the results are as follows:

(a) The night urine will be much less than the total day urine. It is usually 250 to 350 c.c., and will seldom exceed 400 to 500 c.c.; 750 c.c. is the maximum. Its specific gravity will usually be 1.018 or above.

(b) The highest specific gravity recorded for the two-hour day specimens will exceed 1.018 while the difference between the highest and lowest will be not less than 8 or 9 points. If, for example, the most concentrated specimen has a specific gravity of 1.020, the most dilute will be 1.011 or less.

Significance of the Test.—(a) Nocturnal polyuria. The volume of the night urine exceeds 750 c.c. This is usually one of the first and most definite evidences of impaired kidney function. A volume between 500 and 750 c.c. is suspicious, and usually indicates impairment.

(b) Low maximal specific gravity of day or night urine, the highest falling below 1.018.

(c) Fixation of specific gravity, that is, lessened variation in the specific gravities of the two-hour specimens. This is a very important sign of renal functional impairment. In advanced cases the difference between the highest and lowest specific gravities may be only one or two points. As a rule, the level at which the specific gravity is fixed approaches 1.011 as the functional impairment increases and the kidneys lose their ability to concentrate.

The same factors invalidating the concentration test invalidate the Mosenthal test. Occult edema must be carefully excluded before an abnormal result is attributed to renal disease. It is not as sensitive as the concentration tests and adds no information to that obtained by the methods previously described.

UREA CLEARANCE TEST

Principle.—The blood urea clearance is an expression of the number of cubic centimeters of blood that would contain, at the time of study, the quantity of urea removed from the blood by the kidneys in one minute and excreted in the urine during that time. This is usually expressed in terms of percentage of the average

normal, which varies with the body surface area and with the minute volume of urine.

Procedure.—1. The patient is allowed to eat an ordinary breakfast and is given an additional glass of water.

2. The bladder is immediately emptied completely, the specimen being discarded. The exact time is recorded at which the subject finishes voiding, which marks the beginning of Period 1.

3. At the end of approximately one hour the bladder is again completely emptied and the time noted exactly. This marks the end of Period 1. The volume of the entire specimen is carefully measured and it is preserved by storing in the refrigerator.

4. The subject is given another glass of water and blood is removed for urea N determination.

5. At the end of approximately two hours from the beginning of Period 1 the bladder is again completely emptied and the time noted exactly as marking the end of Period 2. The volume of urine is carefully measured and it is preserved in the refrigerator unless examined immediately.

6. Calculate the volume of urine excreted per minute during each period.

7. Determine the urea N concentration of each urine specimen and of the blood sample.

Calculation.—1. If the minute volume of urine exceeds 2 c.c. the expression is that of "Maximum Clearance," the formula being:

$$C_m = \frac{UV}{B} = \text{normally } 64\text{-}99 \text{ c.c. (average 75 c.c.)}$$

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must be taken at each meal; and no food or liquid of any sort may be taken between meals until after 8 o'clock the following morning.

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3. Measure the night urine (8 P.M. to 8 A.M.) and take its specific gravity with an urinometer.

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(b) Low maximal specific gravity of day or night urine, the highest falling below 1.018.

(c) Fixation of specific gravity, that is, lessened variation in the specific gravity of the two-hour specimens. This is a very important sign of renal function impairment. In advanced cases the difference between the highest and lowest specific gravities may be only one or two points. As a rule, the level at which the specific gravity is fixed approaches 1.011 as the functional impairment increases and kidneys lose their ability to concentrate.

The same factors invalidating the concentration test invalidate the Mosenthal. Occult edema must be carefully excluded before an abnormal result is attributed to renal disease. It is not as sensitive as the concentration tests and adds information to that obtained by the methods previously described.

UREA CLEARANCE TEST

Principle.—The blood urea clearance is an expression of the number centimeters of blood that would contain, at the time of study, the quantity removed from the blood by the kidneys in one minute and excreted in the urine during that time. This is usually expressed in terms of percentage of the

Sources of Error.—1. Inaccurate quantity of dye injected. Exactly 1 c.c. of dye must be injected. Since the commercial ampule contains more than 1 c.c., the amount should be measured in an accurately graduated tuberculin syringe.

2. Incomplete or retarded absorption of dye. This is possible whenever the intramuscular route is used. Edema, obvious or preclinical, contraindicates the intramuscular route. Because of the uncertainty of the intramuscular route, it should be employed only when intravenous injection is difficult or technically impossible.

3. Incomplete evacuation of the bladder.

4. Failure to promote diuresis. A urinary output of less than 40 c.c. in any specimen renders the test unreliable.

5. Excessive alkalinization of the specimens. This tends to cause the color to fade.

6. Delay in computing the results when the specimens are alkaline. The specimens should be made acid with acetic acid if delay is necessary.

Normals.—40-60% in first hour, 60-75% in two hours. *Fractional:* at least 25% in 15 minutes.

Significance of Test.—The amount of dye excreted by the kidney depends not only on the functional capacity of the kidney but also upon the amount of dye it receives. Inasmuch as the normal liver excretes about 20% of the dye, in the presence of hepatic functional impairment, renal elimination may be increased and, in some cases, mild renal functional impairment may be masked. On the other hand, heart failure decreases the amount of dye available to the kidney in a given time and hence causes a diminished output. Saline cathartics such as magnesium sulphate delay and alkalinizing substances, such as sodium bicarbonate, tend to increase the excretion of the type. This test should not be made when either of these drugs is used. Slightly lower values are at times found in normal pregnancy. Slightly higher values are obtained in the recumbent than in the upright position.

Its chief field of usefulness is in chronic diffuse glomerulonephritis in which excretion tends to run parallel to the severity of the disease. In the absence of extrarenal factors, 40% indicates impaired function, 30% advanced nephritis and 10% terminal nephritis. The fractional method is the simplest and most sensitive and is the method of choice.

ADDIS SEDIMENT COUNT

Principles.—1. This test is based upon the principle that the number of casts and cells in the urine varies with the type and extent of kidney disease.

2. It is of the utmost importance that the patient clearly understand the conditions under which the specimen must be secured and it is advisable that, in order to prevent errors, the directions be furnished in writing.

Container.—In order that the specimen may reach the laboratory in a suitable condition, the urine should be passed directly into a wide-necked bottle which has been thoroughly cleansed, rinsed with distilled water, dried in the inverted position, and finally rinsed out with a clean solution of formaldehyde,

allowed to drain out for a short time before the bottle is closed with a rubber stopper.

In women, catheterization is essential and it is important that the bladder be *completely emptied.*

Collection of Specimen.—1. The patient is instructed to take breakfast as usual, including coffee, tea, or milk as desired, *but must abstain from all fluids thereafter* during the day and night until the urine has been collected in the morning. In other respects, the diet is unaltered, except that specific instructions are given not to take more fruit than is customary.

2. The time of collection may run from any convenient hour in the evening until the patient arises in the morning covering at least 12 hours.

3. The patient is asked not to void during the afternoon of the day on which the collection is commenced.

4. The first voiding is discarded, all others being passed directly into the container as above described, care being taken to empty the bladder as completely as possible.

5. The time when the collection is begun and ended must be recorded on the bottle.

6. In women, the specimen, as stated above, must be collected by catheter.

Method of Examination.—1. Any uratic precipitate is dissolved by immersing the bottle in warm water; phosphatic turbidity is cleared by the addition of acetic acid, avoiding an excess.

2. Measure the volume (to within ± 2 c.c.), return the specimen to the collecting bottle, stopper, and mix well by repeated inversion.

3. Transfer 10 c.c. to a graduated centrifuge tube with a special narrow tip,[1] and centrifuge 5 minutes at 1800 r.p.m.

4. Decant the supernatant urine and remove the remainder with a capillary pipet.

5. Thoroughly mix the sediment by repeatedly drawing it up and blowing out in the capillary pipet.

6. Place 1 drop of mixed sediment on each cell of a blood counting chamber and count the casts and other formed elements, using a high dry lens.

In normal urines where casts are few, 10 such drops are counted. Where the concentration of formed elements is heavy, the sediment is diluted from 1 to 5 c.c. and 2 drops are counted.

Calculation.—Since in the usual form of blood counting chamber the ruled area is composed of 9 large squares each of which is 1 sq. mm. in area, and since the chamber is 1 mm. deep, the volume contained over the total area is 0.9 c. mm. or 0.0009 c.c.

If 10 areas are counted, the volume of urine represented is 0.009 c.c.

Assuming that 90 casts were found in this volume and that the volume of urine (or 1 per cent sodium chloride solution) in which the sediment was mixed was 0.9 c.c, then $\frac{90 \times 0.9}{0.009} = 9000 =$ number of casts in 0.9 c.c.

[1] Satisfactory tubes may be obtained from A. H. Thomas Co., Philadelphia, Pa.

But as the casts in 10 c.c. are all (presumably) concentrated in the 0.9 c.c. volume, the 9000 represents the number of casts in 10 c.c. of urine.

If the total volume of urine in 12 hours was 300 c.c., then the total number of casts in 12-hour urine is $9000 \times \dfrac{300}{10} = 270.000$.

The following general formula applies to the quantitative determination of all the formed elements: number counted times volume in c.c. in which sediment was mixed divided by volume in c.c. in which count was made multiplied by volume in c.c. per 12 hours divided by 10 equals number in 12-hour urine.

Normal Values and Significance.—1. Normally the casts should be a hyaline and may vary from 0 to 5000: the erythrocytes from 0 to 500.000 and the leukocytes not over 1.000.000. Addis has given the following averages in *diffuse glomerulonephritis* per 12 hours as an example:

	Casts	Erythrocytes	Leukocytes
Acute	690.000	405,000,000	48,000,000
Chronic (active)	1,850,000	34,000,000	14,000,000
Chronic (latent)	48.000	16.000.000	2,400,000
Terminal	398.000	26,400,000	10,000,000

The test is one of the most valuable of available methods of study of renal diseases. It is not strictly a test of renal function but rather an important method for the diagnosis of renal disease by the quantitative study of the morphological characteristics of the urine. In interpreting the findings in terms of renal disease, the presence of lesions of the lower urinary tract must, of course, be carefully excluded. This procedure is particularly valuable in following the progress of the renal lesion in glomerulonephritis and in differentiating this condition from nephrosclerosis. When employed for this purpose the possibility of other forms of kidney disease, such as local infectious processes. tumors, etc., must be excluded.

Gibson's Modification.—1. Allow no fluids on day before test.

2. Empty bladder before retiring, discard urine and note time.

3. Collect all urine passed during the night and also immediately upon awakening. Note time and measure volume.

4. Place one per cent of the volume of mixed urine into two 15 c.c. graduated centrifuge tubes. Centrifuge urine as in Addis method.

5. Remove the supernatant fluid and adjust the volume of sediment so that the volume is

<div style="text-align:center">

0.5 c.c. if test lasted 11-12 hours
0.4 c.c. if test lasted 9-11 hours
0.33 c.c. if test lasted 7-9   hours

</div>

6. Place a drop on a hemacytometer and count the average number in one large square (0.1 c. mm.).

7. Divide the number obtained by 2. This is the number of millions of elements excreted in 12 hours.

CHAPTER VIII

METHODS FOR THE EXAMINATION OF SPUTUM

Principles.—1. In the great majority of instances sputum and bronchial secretions obtained by bronchoscopic drainage are submitted for bacteriological examination, especially for tubercle bacilli.

2. Valuable information is also to be sometimes obtained by careful macroscopical examination, although the finding of Charcot-Leyden crystals, Curschmann's spirals, Dittrich's plugs, etc., is not nearly as frequent as generally believed nor do they possess as much diagnostic value as surmised years ago before bacteriological methods came into general use.

OUTLINE OF COMPLETE EXAMINATION

The complete examination of sputum and bronchoscopic specimens may embrace the following:

1. Physical examination
   - (a) Consistency
   - (b) Color
   - (c) Odor
   - (d) Layer formation
   - (e) Reaction and specific gravity
2. Macroscopic examination aided by a hand lens
   - (a) Curschmann's spirals
   - (b) Dittrich's plugs
   - (c) Broncholiths or lung stones
   - (d) Bronchial casts
   - (e) Fibrin and coagulation
3. Microscopic examination of unstained specimens
   - (a) Elastic fibers
   - (b) Curschmann's spirals
   - (c) Charcot-Leyden crystals
   - (d) Pigmented or "heart-failure" cells
   - (e) Myelin globules
   - (f) *Actinomyces hominis* (ray fungus)
   - (g) Molds and yeasts
   - (h) Animal parasites
4. Microscopic examination of stained specimens
   - (a) Tubercle bacilli
   - (b) Pneumococci; streptococci; staphylococci; etc.

  (c) Spirochetes and fusiform bacilli
  (d) Pus
  (e) Eosinophils
  (f) Erythrocytes
  (g) Epithelium
5. Chemical examination
  (a) Albumin
  (b) Of *saliva* for nitrogenous substances and especially urea

COLLECTION

1. For tubercle bacilli, a twenty-four-hour specimen is recommended. For pneumococcus typing a single specimen may suffice. Special methods of collection for these and other bacteriological examinations are given in Chapter XV.

2. For tubercle and other general examinations sterile containers are unnecessary but they should be clean.

3. Morning sputum is recommended if single specimens are to be examined.

4. As a general rule it is advisable for the patient to wash the mouth and teeth before collection, especially of single specimens, to avoid extraneous contamination as far as possible.

5. A preservative like phenol or tricresol may be used for specimens to be examined for tubercle bacilli. Otherwise they should be avoided.

6. Patients should be instructed to distinguish between saliva and sputum. As far as possible the former and postnasal secretions should be avoided and deep bronchial secretions collected.

7. The container should be a small, wide-mouthed bottle or vial fitted with a light stopper to prevent outside contamination and spilling, and to permit sterilization. Well-constructed paper sputum cups are acceptable. Paper napkins are unsuitable except for pneumococcus typing.

PHYSICAL EXAMINATION

1. If collected in a graduated glass, record the amount and whether or not layers are formed. Some sputa show a striking tendency to form three layers, especially in bronchiectasis, gangrene, and abscess of the lung.

2. Record the appearance as:

  (a) Mucoid (glairy, transparent and tenacious)
  (b) Purulent (pus or mucus and pus)
  (c) Serous (colorless or yellow; frothy)
  (d) Bloody (streaked; rusty; "prune-juice"; pure blood)
  (e) Combinations of above
  (f) Color (yellow; gray; greenish; rusty or brown; blackish; reddish or
    combinations)
  (g) Odor (putrid; sweetish; cheesy, or none)
  (h) Layers if kept in a tall glass for some hours

(i) Coagulation in case of bronchoscopic drainage specimens (complete; partial)

(j) Reaction (of little importance)

(k) Specific gravity (only upon special request)
  Mucoid sputa: usually 1.001 to 1.008
  Purulent sputa: usually 1.015 to 1.06
  Serous sputa: usually 1.037 and higher

MACROSCOPIC EXAMINATION

1. Pour a portion of the sputum into a Petri dish or between two large panes of glass (former less messy and preferred) to give a thin layer.

2. Carefully examine against a black background with the aid of a hand lens.

3. Pick out portions for microscopic examination.

4. Examine for:

FIG. 119.—CURSCHMANN'S SPIRAL, SHOWING CENTRAL THREAD (Wood)

(a) Curschmann's spirals, which are yellowish-white masses having a twisted appearance surrounded by mucus (Fig. 119)

(b) Bronchial casts (Fig. 120)

(c) Dittrich's plugs, which are yellowish-white bodies varying in size from a millet seed to a bean and having a very putrid odor

(d) Lung stones or broncholiths

MICROSCOPIC EXAMINATION OF UNSTAINED SPUTUM

1. Carefully select suspicious particles and place on slides; cover with glasses.

2. Examine with low and high lens with the light well cut down as in the examination of urine sediment.

3. Examine for:

(a) Elastic fibers (Fig. 121)

(b) Pigmented cells ("heart-failure cells"): large epithelial cells containing hemosiderin, a brownish pigment. To demonstrate more clearly apply a drop of a 10 per cent solution of potassium ferrocyanide for a few minutes, followed by a drop of a normal solution of hydrochloric acid. Iron-containing pigment becomes a blue color (Prussian blue reaction)

(c) Curschmann's spirals

(d) Charcot-Leyden crystals (Fig. 122)

(e) Myelin globules, which possess little or no significance except that they may be confused with blastomyces (Fig. 123)

(f) *Actinomyces hominis* or "sulphur granules" (Fig. 124)
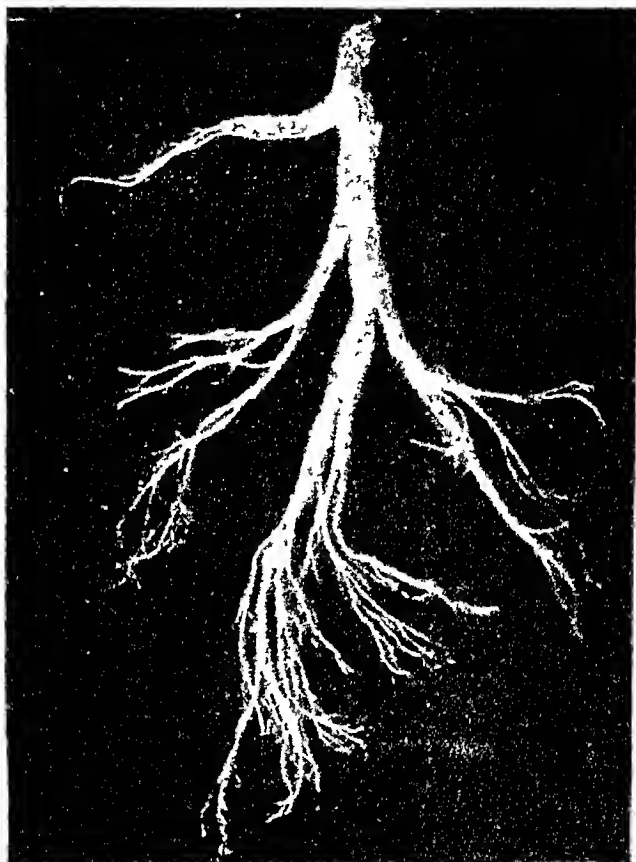
Fig. 120.—Bronchial Cast (Wood)

(g) Molds and yeasts as *Aspergillus fumigatus, Oïdium albicans,* the fungus *Mycoderma,* etc.

MICROSCOPIC EXAMINATION OF STAINED SPUTUM

1. For tubercle bacilli, pneumococci, spirochetes, etc., see Chapters XVIII and XIX.

2. For eosinophils. pus, blood, etc., prepare thin smears on slides and stain with Wright's stain as described in Chapter IV for the staining of blood smears.

3. Examine for:

(a) Eosinophils
(b) Polymorphonuclear leukocytes (pus cells)
(c) Erythrocytes
(d) Kinds of epithelial cells
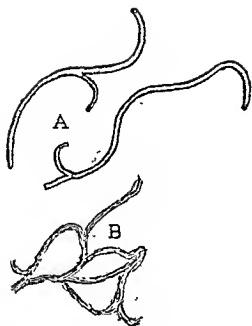
FIG. 121.—YELLOW ELASTIC TISSUE

A. Single fibrils, highly magnified. B. Alveolar elastic tissue. (Morris)

FIG. 122.—CHARCOT-LEYDEN CRYSTALS (Morris.)
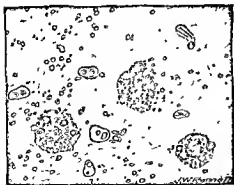
FIG. 123.—MYELIN GLOBULES. × 350

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)
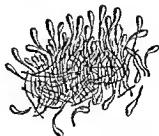
FIG. 124.—ACTINOMYCES HOMINIS, SHOWING CLUB-SHAPED EXTREMITIES TO THE RAYS (Wood)

4. *Paragonimus westermanii,* called the "lung fluke," is a common parasite of man in Japan, China and Korea. It inhabits the lung, causing the formation of small cavities. Moderate hemoptysis is the principal symptom. Ova are readily found in the sputum (Fig. 125); the flukes themselves are seldom seen, except postmortem. They somewhat resemble a coffee bean in size and shape. They are faintly reddish-brown in color, egg-shaped, with the ventral surface flattened, and measure 8 to 10 by 4 to 6 millimeters. The ova are thin-shelled, operculated, brownish yellow, and measure from 87 to 100 by 62 to 66 micra.

FIG. 125.—SPUTUM OF MAN CONTAINING EGGS OF THE LUNG FLUKE, ABOUT ×133. (after Manson)

(Reproduced from *Bulletin No. 17, Hygienic Laboratory of the U.S.P.H. and Marine Hospital Service.*)

There are two intermediate hosts, a mollusk in which the cercariae are formed, and a fresh-water crab (a common article of food in Japan) in which they encyst. The encysted forms have also been found in fresh-water snails.

According to Ward, three distinct species have been confused under the name *Paragonimus westermanii:* the original form, *Paragonimus westermanii,* found in the tiger; the American lung fluke, *Paragonimus kellicotti,* thus far found only in cat, dog and hog; and the Asiatic lung fluke of man, *Paragonimus ringeri* (Fig. 126).

CHEMICAL EXAMINATION

FIG. 126.—OVUM OF PARAGONIMUS RINGERI. ×400 (After Emerson.)

Test for Albumin.—1. Use fresh sputum, as decomposed specimens are unsatisfactory.

2. To 10 c.c. add 20 c.c. of a 3 per cent solution of acetic acid in water.

3. Shake vigorously and filter through paper.

4. Test the filtrate by the Esbach method for the quantitative determination of albumin in urine as described in Chapter VI.

5. In tuberculosis and pneumonia the albumin varies from 0.1 to 0.3 per cent or 1 to 3 grams per liter. In chronic bronchitis and asthma only traces are found.

Test for Occult Blood.—The benzidine or other methods described in Chapter XII for occult blood in feces may be employed.

Test of Mercury-Combining Power of Saliva for Nitrogenous Substances and Especially Urea (Hench and Aldrich).—*Reagent.*—An accurately prepared 5 per cent solution of chemically pure mercuric chloride in distilled water.

Collection of Saliva.—The mouth is well rinsed with water. Chewing of a small piece of paraffin or holding a small marble in the mouth will favor the flow of saliva, but this is not necessary. The saliva is collected in two portions of about 8 c.c. each. The first of these carries off food particles and epithelial débris and is discarded. The second is used for the titration. It need not be filtered.

Method.—1. By means of a pipet transfer 5 c.c. of the saliva to a small flask or beaker.

2. Add 5 per cent solution of mercuric chloride from a buret or pipet a few drops at a time, with constant stirring, until a drop of the fluid, when added to a drop of saturated solution of sodium carbonate on a white porcelain plate, gives a definite reddish-brown color. The color should appear within about three seconds. If it develops more slowly, the end-point is near, but not yet reached, and a few additional drops of the bichloride must be added.

3. When the end-point is reached note the number of c.c. of mercuric chloride solution which have been added. This is the number of c.c. which would be required for 100 c.c. of saliva. Record this as the "mercury-combining index."

Hench has found the mercury-combining index in normal persons to lie between 30 and 50 for 100 c.c. of saliva. When there is retention of urea in the blood the index rises with the blood urea, although it lags a little behind. The probable blood urea may be roughly calculated as follows:

$$1.43 \times \text{salivary index} - 34 = \text{probable blood urea in milligrams}$$
$$\text{for each 100 c.c.}$$

Example: Suppose the salivary index were 100. Then $1.43 \times 100 - 34 = 109$ milligrams urea in 100 c.c. of blood.

CHAPTER IX

METHODS FOR THE COLLECTION AND EXAMINATION OF STOMACH CONTENTS

INTRODUCTION OF THE STOMACH TUBE

1. The Rehfuss or other standard small gastro-duodenal tube may be used. Lyon tube is recommended.

2. If the patient is neurotic or has marked pharyngeal hyperesthesia, swab the fauces with a 2 per cent solution of cocaine hydrochloride.

3. The patient should be seated with the head tilted slightly forward and the clothing protected with towels or an apron.

4. Place the tip of the tube on the tongue held well out, and pass it back to the throat. Then the patient is encouraged to swallow quickly upon removal of the fingers, while the tube is slowly fed into the mouth. The patient should keep the lips closed and breathe deeply.

After slight discomfort in the pharynx and passage of the tube to the level of the cricoid cartilage, practically no discomfort is felt. If the patient has difficulty, he may be given a measured portion of the water of the test meal to swallow along with the tube, as this carries the latter to the stomach with a minimum of discomfort. The amount so given is, of course, considered in the final calculation.

It is sometimes advantageous to push the tube over to the side of the mouth, back of the teeth and passing behind the last molar tooth, just as soon as the tip has passed into the esophagus. The patient should keep the mouth closed. This causes less reflex gagging and less chance of contaminating the stomach contents with mucus from the nose and pharynx.

METHODS OF EXAMINATION

1. Either of two methods may be employed: (a) The older consists in giving an Ewald breakfast and removing the stomach contents one hour later. (b) By the newer fractional method of Rehfuss, the tube is introduced into the fasting stomach, the residuum removed, a meal given with the tube in situ and fractions removed every fifteen minutes for one or two hours or longer.

2. If the older method is employed, the Töpfer method of chemical analysis may be used; if the fractional method is used each portion is examined for free and total acidity, protein and any other tests decided upon.

3. The newer fractional method is recommended because it permits of a study of the gastric residuum, gives much more accurate information of the chemical

and enzymic activities of the stomach and permits of an estimation of gastric emptying. Both methods require fasting for 12 hours.

GASTRIC ANALYSIS BY THE FRACTIONAL METHOD

Procedure.—1. Removal of the residuum, feeding the test meal and removing samples every fifteen minutes for two hours.

2. Macroscopic examination of samples.

3. Determination of total acidity of each sample.

4. Determination of free hydrochloric acid of each sample.

5. Testing for lactic acid, especially when there is no free hydrochloric acid.

6. Testing for enzymes, especially when there is no free hydrochloric acid.

7. Testing for occult blood.

8. Microscopical examination of the residuum and of those samples containing bile.

REMOVING FRACTIONS OF STOMACH CONTENTS

Principles.—If properly carried out this constitutes one of the most valuable gastro-enterological examinations. Improperly and indifferently performed, it is of little value. The following classification represents a summary of the information which may be obtained by this method.

1. Measure of gastric work:
   (a) Secretory function: acid and enzyme determination.
   (b) Motor function: examination of fasting residuum for food and determination of the emptying time of the Ewald meal.

2. Indication of intragastric disease:
   Addition to the gastric secretion of abnormal elements such as: blood (microscopic or occult), pus, mucus (of stomach origin), exfoliated epithelium, bacterial colonies, tissue fragments and foreign bodies.

3. Indications of extragastric disease:
   (a) Extragastric type of acidity curve (indirect evidence).
   (b) Products of extragastric disease (direct evidence):
      (1) Swallowed pus, blood or mucus as indicative of lesion higher up.
      (2) The presence of bile in the stomach residuum or constant regurgitation of bile during the analysis is suggestive of abnormal motor activity of the pylorus and duodenum. Pathological products in this bile residuum as blood, pus, mucus, exfoliated gall tract epithelium, cholesterin crystals, and numerous bile-stained organisms may be due to disease beyond the pylorus.

Procedure.—1. Patient may be instructed to take a meal twelve hours preceding the examination. A dish of rice and raisins, a meat sandwich and 30 raisins or a meal to include 4 stewed prunes will be satisfactory as the plan is to ingest some heavy cellulose which will be readily recognized in the gastric residuum the following morning.

2. The teeth should not be brushed on the morning of the examination to exclude any possibility of swallowing blood.

3. A gastroduodenal tube is passed to a point 56 cm. from the lips as described above. Note should be made of the amount of gagging and retching which accompanies the passage of the tube. The reason for this observation is twofold. When there is considerable gagging and retching, bile is frequently regurgitated into the stomach. Recently regurgitated bile will be of lemon yellow tint. Bile, after being in the stomach, becomes of a greenish turbid hue due to the action of the hydrochloric acid. Consequently the finding of some lemon yellow bile in the stomach is usually due to regurgitation during the passage of the tube and is of no significance.

4. Extract all of the residuum with as little traumatism as possible. Measure the quantity and save for examination.

5. Give the Ewald test meal with the tube *in situ.* *i.e.,* 35 grams of bread without the crust or a shredded wheat biscuit and 350 c.c. of water. Instruct the patient to thoroughly masticate the bread or shredded wheat before swallowing.

6. Any saliva which forms in the mouth after the meal is finished is to be expectorated into a basin and must not be swallowed. The irritation of the tube in some patients will cause an almost constant flow of saliva. If it is swallowed it will greatly reduce the stomach acidity. The amount of saliva expectorated in the two-hour period is measured and recorded. Normally from 25 to 50 c.c. or less will be expectorated. If 200 c.c. or more are obtained, hyperptyalism is present, which may constitute evidence of vagotonia. The saliva may be examined microscopically and chemically for enzyme content to eliminate any disease of the salivary glands if there are pointings in that direction.

7. An extraction should be made every 15 minutes or the test may be simplified by making extractions every 30 minutes. An amount approximating 10 c.c. should be withdrawn with each extraction in order that a sufficient quantity of juice will be available for any special examinations which may be desired. In making the extractions, as little traction on the syringe as possible is desirable. If considerable suction is made on the syringe when the tube is collapsed, the mucosa will be traumatized and occult or gross blood will be the result. If such traumatism has been caused it is well to note the fact so that the occult blood reactions may be properly interpreted. It will be remembered that one of the important points to be learned from the fractional examination is the motor power of the stomach. With this in mind there are two methods of terminating the examination:

The method recommended is to withdraw specimens every 15 minutes for a period of 2 hours from the time of ingestion of the meal. Then at the end of two hours the stomach is emptied by the syringe and the amount of chyme left in the stomach is measured and recorded. A food residue of much over 5 c.c. at the end of the two-hour period is indicative of delay in emptying. The amount of the residue will indicate the degree of hypomotility. After the stomach has been emptied with the syringe, a lavage of 250 c.c. of water should be made. Allow that amount of water to run in and out. This will give positive information as to

the emptiness of the stomach and will act as a check on the emptying of the stomach with the syringe.

The other method consists in withdrawing 15-minute extractions until the stomach is completely empty to get the measure of motility of the stomach. This procedure takes considerably longer in the hypomotile cases and is not necessary if one gages the amount of food remaining in the stomach at the end of 2 hours and learns to compute the degree of motor impairment. The first procedure also has the advantage that it requires less time and is less tiring to the patient.

8. The following data should be recorded concerning each sample which is extracted:

(a) A record is made of the amount of secretion which is withdrawn at each extraction. It is well not to remove more than 10 c.c. each time in order to allow the bulk of the food to be acted upon by the stomach. At the end of two hours the stomach is emptied. In order to be sure that the stomach is empty the patient should be told to assume various positions, aspiration being done in each position, i.e., on the back, abdomen, right side and left side. The amount withdrawn at this last extraction is also recorded. The sum of the amounts withdrawn throughout the examination subtracted from the amount of liquid given with the meal will represent roughly the amount of fluid which has passed through the pylorus in two hours. This, of course, does not allow for the amount of juice secreted.

(b) A rough estimation of the amount of food withdrawn with each extraction should be made. This will give some idea of the motility of the stomach. If after an hour or an hour and one half no food is obtained with the extraction, rapid emptying must be suspected, providing the tube is in the right position. If a residue at the end of two hours shows more than 10 c.c. of food at the bottom of the glass, the stomach emptying power is delayed. In the presence of an abnormal amount of food at this time, it is often impossible to extract all of the stomach residue. For this reason it is well to routinely lavage the stomach with 250 c.c. of water after the last extraction has been made. The amount of food residue in the return wash is recorded. Finding more than 10 c.c. of food in the return wash and the last extraction combined indicates hypomotility. The more above 10 c.c. the greater the degree of delayed emptying or obstruction. This is one of the most important steps in fractional gastric analysis. Too often in routine hospital and *office work the examiner is satisfied with a report of the acidity alone, which con-* stitutes only a minor part of the information which may be obtained if this test is carried out properly. This method of testing the emptying time of the stomach is just as accurate as the six-hour barium x-ray meal and can be carried out often when an x-ray examination is not feasible. It checks with the x-ray method in the severe cases of obstruction and is a more refined and accurate method where the delay is slight.

(c) A record is made of the color of each extraction. Bile, regurgitating into the stomach, gives the stomach juices a yellow or greenish tint. The amount of bile should be recorded. It should be gaged by the intensity of the color, and recorded as plus 1, 2, 3 and 4. Bile may be found in the normal stomach.

Gross blood in the stomach often gives the contents a diffuse brown tint. A sufficient amount of blood to cause its macroscopic appearance in the stomach juice means serious disease of or about the stomach, unless it has been swallowed. Bright red blood may be due to trauma from the tube tip. This occurs more readily in the presence of disease of the gastric mucosa.

MACROSCOPIC EXAMINATION

1. The *amount* of juice in the fasting stomach should not exceed 50 c.c. It normally varies from 15 to 40 c.c. An increase above 50 c.c. means either hypersecretion, hypomotility or obstruction. There should be no food in the eight-hour fasting stomach. It should be remembered that a very nervous patient may swallow very large amounts of saliva during the passage of the tube and greatly increase the amount of the residuum in that manner. However, it is not difficult to differentiate mouth mucus from stomach juice.

2. *Bile* in the fasting residuum, if found repeatedly and in large quantities, is indicative of some disease if the patient is "tube broken." The commoner conditions to be thought of in this connection are hyperchlorhydria, bile tract disease, duodenal ulcer, duodenitis and rigid pylorus from stenosis or adhesions. This should not be confused with the recently regurgitated bile due to retching in the taking of the tube. Such recently regurgitated bile is lemon yellow in color and forms a yellow foam when shaken. On the other hand, bile which has resided in the stomach for a considerable period is distinctly green and turbid. Some observers claim that bile is normally found in the gastric residuum. Large quantities of turbid green bile in a retentive stomach are often due to small bowel obstruction below the ampulla of Vater.

3. *Mucus,* if of stomach origin and present in considerable quantity, is indicative of catarrhal inflammation of the stomach. It is important, in this connection. to be able to differentiate stomach mucus from swallowed mucus. If it originates in the stomach it is flaky with particles suspended in the gastric juice. On the other hand, swallowed mucus is generally in large stringy masses and floats on the top of the gastric juice.

4. *Pus,* present in sufficient quantities to be recognized, is very rare. Such a finding would point toward acute or chronic diffuse suppuration of the stomach, abscess of the stomach or rupture of an abscess into the stomach (subphrenic, retroperitoneal, pancreatic, biliary, hepatic or splenic in origin).

5. *Odor* of the gastric residuum is not usually of much importance. In cancer and severe catarrhal gastritis, a pungent disagreeable odor may be noted. Colon bacillus infection of the stomach, of course, has a characteristic odor but it is very rare. The odor of feces is usually due to bowel obstruction or to gastrocolic fistula. A very unpleasant more or less characteristic sour odor is present in fermentation of the stomach contents.

6. *Blood,* if grossly recognized in the fasting residuum, is of the utmost importance. More than 50 per cent of all cases of cancer of the stomach will show small quantities of blood in the stomach. As a rule it is changed by the stomach

juices and somewhat resembles coffee grounds. The average cancer does not bleed profusely but it bleeds constantly, hence there is considerable changed blood in the stomach at any given time. Gross blood is not as common in ulcer of the stomach. Occasionally, however, it will be present in very large quantities and is often bright red in color. Esophageal varices, sclerosed stomach vessels, erosions and severe infections also give rise to free blood in the stomach but more rarely. Duodenitis, duodenal ulcer, pancreatic or biliary tract carcinoma may cause the appearance of free blood in the stomach. Injury to the mouth or esophageal mucosa in swallowing the tube and blood from the lungs and nose should be borne in mind. '

7. **Food in the fasting stomach** may be indicative of atony, dilatation, or ptosis of the stomach or, what is more likely, pyloric stenosis, pyloric or duodenal adhesions, pylorospasm, mass at or near the pylorus or pressure from without. The amount of food present and the constancy of its presence will help to decide whether one of the more serious causes is the most likely.

DETERMINATION OF FREE HYDROCHLORIC AND TOTAL ACIDITY

Procedure for Free Hydrochloric Acid.—1. Place 5 c.c. of strained or filtered gastric juice in a porcelain dish. When the above amount is not available use 1 c.c. to which 4 c.c. of distilled water may be added.

2. Add 2 drops of a 0.5 per cent solution of dimethyl-aminoazobenzene in 95 per cent alcohol (Töpfer's indicator). If free acid is present a red or orange color develops (see C in plate V).

3. Titrate with N/10 or N/20 sodium hydroxide until the red or orange color disappears (if 1 c.c. of gastric juice is used it is advisable to use N/20 sodium hydroxide) and a yellow color develops (see C' in plate V).

4. Note the amount of sodium hydroxide used and calculate the number of c.c. of N/10 sodium hydroxide required for the neutralization of 100 c.c. of gastric juice. The result is always expressed as the number of c.c. of N/10 sodium hydroxide required to neutralize 100 c.c. of gastric juice. If 5 c.c. of gastric juice is titrated with N/10 sodium hydroxide, then multiply the amount used by 20. If 1 c.c. of gastric juice is titrated with N/20 sodium hydroxide, then multiply the amount used by 50.

5. The total acidity of this same specimen can be determined by continuing the titration after the addition of phenolphthalein as an indicator following the procedure given below for determining total acidity, beginning with step 2.

Procedure for Total Acidity.—1. Place 5 c.c. of strained or filtered gastric juice in a porcelain dish. When the above amount is not available use 1 c.c. to which 4 c.c. of distilled water may be added.

2. Add 2 drops of a 1 per cent solution of phenolphthalein in 95 per cent alcohol. In the presence of acid there is no change in color (see A plate V).

3. Titrate with N/10 or N/20 sodium hydroxide until a bright red color is obtained which persists (see A' in plate V).

PLATE V
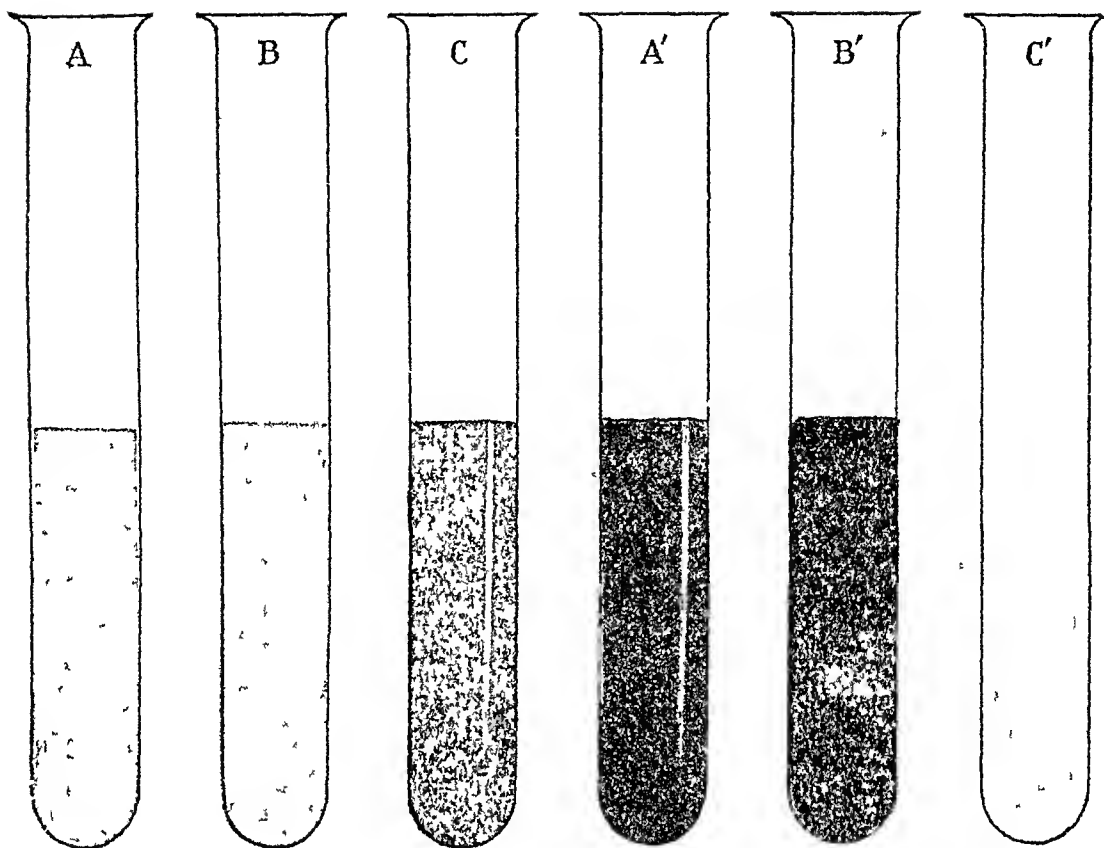
A, gastric fluid to which a 1 per cent solution of phenolphthalein has been added; B, gastric fluid to which a 1 per cent solution of alizarin has been added; C, gastric fluid to which a 0.5 per cent solution of dimethylamino-azobenzol has been added; A', A after titration with a decinormal solution of sodium hydroxide; B', B after titration with a decinormal solution of sodium hydroxide; C', C after titration with a decinormal solution of sodium hydroxide (Boston). (From Todd and Sanford's *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

4. Note the amount of sodium hydroxide used and calculate the number of c.c. of N/10 sodium hydroxide required to neutralize 100 c.c. of gastric juice.

If 5 c.c. of gastric juice is titrated with N/10 sodium hydroxide, then multiply the amount used by 20. If 1 c.c. of gastric juice is titrated with N/20 sodium hydroxide, then multiply the amount used by 50.

When the free and total acid are both determined with the same specimen (one as a continuation of the other), then the amount of N/10 sodium hydroxide used for both procedures are combined and used for calculating the total acid.

The normal range for total acidity is from 40 to 60.

DETERMINATION OF COMBINED HYDROCHLORIC ACID AND ACID SALTS

Procedure.—1. Place 5 c.c. of gastric juice in a porcelain dish.

2. Add 3 drops of a 1 per cent solution of sodium alizarin sulphonate in water (see B in Plate V). This indicator reacts to both free hydrochloric and acid salts.

3. Titrate with N/10 sodium hydroxide until a *distinct violet color* is obtained (see B' in Plate V). It is difficult, without practice, to determine when the right color has been reached. A reddish-violet first appears (Plate V, B'). The tendency is to add too much sodium hydroxide. The color of the end-reaction can be imitated fairly well by adding 2 or 3 drops of the indicator to 5 c.c. of a 1 per cent solution of sodium carbonate.

4. Take the buret reading and multiply by 20 to determine the free acidity of 100 c.c.

Subtract the free acidity (alizarin indicator) from the total acidity (see page 204) to obtain the so-called *combined hydrochloric acid* (a protein salt of the acid).

Subtract the free hydrochloric acid (see page 204) from the free acidity (alizarin indicator) to obtain the *acid salts* and organic acids.

CURVES OF TOTAL ACIDITY AND FREE HYDROCHLORIC ACID

1. **Normal Curve.**—The apex of the curve is reached at from sixty to ninety minutes after the Ewald meal and a return to within 20 degrees of the fasting acidity occurs in two hours. The apex values average: free hydrochloric acid, 45 to 55; total acidity, 55 to 65. This is the most frequent type of curve found in normal individuals, but there is often a wide deviation from this normal contour in otherwise normal people.

2. **Hyperchlorhydria Curve.**—The contour of this curve conforms to the normal curve except that the acid values are much higher. The fasting acidity will be free hydrochloric acid, 40; total acidity, 55 or higher. The apex of the curve will show: free hydrochloric acid, 75; total acidity, 90 or higher. This type of curve is occasionally found in normal individuals, but more commonly in cases of early or incipient peptic ulcer.

3. **Stepladder Curve.**—Described by Lyon and Best and supposed to be of serious diagnostic import. According to these observers it is only seen in cases

of active ulcer and usually precedes or follows a hemorrhage. The smooth ascent of the curve is broken by drops in both free and total acid, unassociated with biliary regurgitation, followed by a rise to a still higher level than that preceding the drop. The actual acid values are usually much above normal in this type of curve.

4. **Extragastric Curve.**—The acid values, instead of starting to recede after sixty or ninety minutes, continue to rise, the last extraction having the highest acid values. This type of curve is commonly seen in duodenal ulcer.

5. **Delayed Digestion Curve.**—Also sometimes called psychical achylia. The acid values remain very low until 45 or 60 minutes after the meal, when the acid rises and goes through a normal curve. The delayed secretion is probably due to the absence of the cephalic phase of gastric secretion. This type of curve is often seen in patients who have great difficulty in swallowing and retaining the tube because of gagging.

6. **Hypochlorhydria Curve.**—Hypochlorhydria may be benign or due to carcinoma of the stomach. There may be no free hydrochloric acid or but small amounts, being under 40 throughout the two hours. The total acidity is low, being under 40, or reaching about 40 toward the end of the first hour.

HISTAMINE TEST FOR TRUE AOHYLIA

This is conducted in cases of achylia by injecting *subcutaneously* 0.00025 gram (0.25 c.c. of 1:1000 solution of histamine) after the introduction of the tube and the evacuation of the residuum, simultaneously with the ingestion of the meal. It may be injected just after the ingestion of any standard test meal. A glass of water is commonly used since the test is usually used after a standard fractional gastric analysis has suggested an achylia.

Specimens are removed at fifteen-minute intervals as for fractional analysis, and titrated for total acidity and free hydrochloric acid. If the stomach is capable of producing hydrochloric acid, the glands are stimulated by the reaction produced by the histamine. If acid and enzymes are absent after histamine injection a true achylia is stated to be present.

GASTRIC ANALYSIS BY THE TÖPFER METHOD

Procedure.—1. Feed the Ewald test meal of two pieces (35 grams) of toast and 8 ounces (250 c.c.) of tea.

2. *One hour* later remove the *entire* stomach contents.

3. Make the macroscopic examination as described on page 203 for amount, bile, mucus, pus, odor and gross blood. The amount varies under normal conditions between 50 and 100 c.c. In cases of hypersecretion or defective motility, 200 to 300 c.c. may be found. Very large amounts, as 500 to 3000 c.c., are indicative of dilatation and suggestive of pyloric stenosis.

4. Make chemical analyses for (a) total acidity, (b) free acidity (free hydrochloric acid and acid salts), and (c) for free hydrochloric acid with subsequent

calculations of (d) combined acidity, and (e) acidity due to organic acids and acid salts.

5. In addition, test for lactic acid, occult blood, and protein, and make a microscopic examination.

DETERMINATION OF LACTIC ACID

Kelling's Test.—This is a fairly satisfactory color test depending upon the formation of ferric lactate.

1. Fill a test tube with water.

2. Add 2 drops of a 10 per cent solution of ferric chloride to give a faint canary yellow. Mix well.

3. Pour one-half into a second tube for a control.

4. To one tube add 1 c.c. of strained stomach juice.

5. If lactic acid is present a deep yellow color develops.

Strauss's Test.—This method depends upon extracting the lactic acid with ether and thereby removing such disturbing factors as hydrochloric acid, digestion products, etc. It also gives an approximate idea of the amount.

1. Place 5 c.c. of strained gastric juice into a small separatory funnel with a stopper (Fig. 127).

2. Add 20 c.c. of ether and shake thoroughly.

3. After separation of the ether, allow the fluid to run out except the upper 5 c.c. of the former.

4. To the ether extract, add 20 c.c. of distilled water and 2 drops of a 10 per cent solution of ferric chloride.

5. Shake gently.

6. A slightly green color is produced in the presence of 0.05 per cent lactic acid whereas 0.1 per cent yields a very intense yellowish-green color.

Uffelmann's Test.—1. Add 5 c.c. of filtered gastric juice to 50 c.c. of ether and shake thoroughly for ten minutes.

2. Collect the ether and evaporate to dryness.

Fig. 127.—Separatory Funnel

3. Dissolve the residue in 5 c.c. of water and add to the reagent:

Mix 0.2 c.c. of concentrated solution of phenol and 0.2 c.c. of aqueous solution of ferric chloride. Dilute with water until the mixture assumes an amethyst-blue color.

4. If lactic acid is present the solution will develop a canary yellow color.

5. This test may be applied to gastric juice direct without first extracting with ether but this is not as reliable as the above because of interfering substances, such as phosphates, sugars, etc.

DETECTION OF OCCULT BLOOD

Benzidine Test.—1. Place a knife point full of benzidine in a test tube (use Merck's blood-testing benzidine).

2. Dissolve in 2 c.c. glacial acetic acid. Warm the solution if necessary.

3. Add 2 c.c. of hydrogen peroxide (3%).

4. Add 1.0 c.c of the fluid to be tested.

5. A positive reaction is indicated by the appearance of a blue or green color.

6. This test is stated to detect blood when present in a dilution of 1 : 3,000,000.

Orthotoluidine Test.—1. In a test tube mix 1 c.c. of reagent with 1 c.c. of gastric juice and 1 c.c. of 3% hydrogen peroxide.

<div align="center">REAGENT</div>

Orthotoluidine .... 4.0 c.c.
Glacial acetic acid q.s. .... 100.0 c.c.

Dissolve. Keeps for a month without loss of delicacy.

2. In the presence of blood a bluish color develops (sometimes rather slowly) which persists for some time (several hours in some instances).

Other methods are the aloin, guaiac and Gregersen's tests described in the chapters on examination of urine and feces.

<div align="center">DETECTION OF BILE</div>

Principle.—The following method is based upon the oxidation of the bilirubin with nitric acid to form biliverdin (green).

Procedure.—1. Place about 1 inch of powdered ammonium sulphate in a test tube and add 10 c.c. of gastric juice.

2. Shake vigorously for a minute.

3. Add 3 c.c. of acetone and thoroughly mix by inverting the tube six times (do not shake).

4. Allow the acetone to separate.

5. Allow a drop of nitric acid to flow down the side of the tube.

6. A green color is a positive reaction. If too much acid is used the biliverdin will be oxidized to a purple or red.

7. If the gastric juice is of a deep green color, dilute 4 or 5 drops with 10 c.c. of water and proceed as above.

<div align="center">DETECTION OF PEPSIN</div>

Principles.—Pepsinogen is normally secreted by the stomach. It has no digestive power until transformed into pepsin by free hydrochloric acid (to a lesser extent by organic acids and the protein salt of hydrochloric acid). Its presence is detected by the digestion of egg albumin.

Qualitative Procedure.—1. Place 25 c.c. of gastric juice in a small flask. If the specimen does not contain free hydrochloric acid, add a few drops of 10 per cent hydrochloric acid.

2. Place in the flask with the gastric juice a disk of coagulated egg albumin. Stopper and place in an incubator at 37° C.

3. If pepsin is present the disk will begin to swell in from one-half to one hour and dissolve in about three hours.

PREPARATION OF EGG ALBUMIN DISKS.—(*a*) Boil an egg very slowly until the albumin is distinctly coagulated.

(*b*) Cut the albumin into small cylinders about 5 millimeters in diameter.

(*c*) Section the cylinders into small disks about 1 millimeter thick.

(*d*) The disks can be preserved in glycerin until needed, but should be washed in water before using.

Quantitative Procedure (Method of Mett Modified by Nirenstein and Schiff).—1. Introduce into a small Erlenmeyer flask 1 c.c. of gastric juice and 15 c.c. of N/20 hydrochloric acid (0.18 per cent hydrochloric acid).

2. Add two Mett tubes prepared as indicated below, stopper the flask to prevent evaporation and place in an incubator at 37° C. for twenty-four hours.

3. By means of a low-power microscope and a millimeter scale (graduated to half millimeters) determine accurately the length of the column of albumin digested at each end of the tubes. It is well to run the determination in duplicate, in which case the result is the average of the eight figures obtained.

4. Ordinarily from 2 to 4 millimeters of albumin are digested by normal human gastric juice.

5. The peptic power is expressed as the square of the number of millimeters of albumin digested. This is based on the Schütz-Borissow law that the amount of proteolytic enzyme present in a digestion mixture is proportional to the square of the number of millimeters of albumin digested. Therefore a gastric juice which digests 2 millimeters of albumin contains four times as much pepsin as one which digests only 1 millimeter of albumin. For example, if the microscopic reading gives on an average 2.2 millimeters of albumin digested, the pepsin value for the diluted juice would be $2.2 \times 2.2 = 4.84$ and for the pure undiluted juice, $4.84 \times 16 = 77.44$.

PREPARATION OF METT TUBES (CHRISTIANSEN's METHOD).—The liquid portions of the whites of several eggs are mixed and strained through cheesecloth. The mixture should be homogeneous and free from air bubbles. It is best to allow the egg white to stand for two or three hours in a vacuum desiccator to remove air more completely. A number of thin-walled glass tubes of 1 to 2 millimeters internal diameter are thoroughly cleaned and dried and cut into lengths of about 10 inches. These are sucked full of the egg white and kept in a horizontal position. Into a large evaporating dish or basin 5 to 10 liters of water are introduced and heated to boiling. The vessel is then removed from the fire and stirred with a thermometer until the temperature sinks to exactly 85° C. The tubes filled with egg white are immediately introduced and left in the water until it has cooled. The tubes thus prepared are soft boiled, more easily digested than hard-boiled tubes, and free from air bubbles. The ends are sealed by dipping in melted paraffin or sealing wax (preferably the latter), and the tubes can be kept thus for a long time. When ready for use, mark with a file and break into pieces about 3 or 4 inches long. After cutting, the tubes should be immediately introduced into the

digestion mixture or may be kept a short time under water. Tubes whose ends are not squarely broken off must be rejected.

The digestibility of different egg whites varies widely. Hence, in making up a new set of tubes, if we wish our results to be comparable these tubes must be standardized against those first prepared. This may be done by running simultaneous tests with tubes from the two series, using the same gastric juice and comparing the lengths of the column digested in each case. Christiansen's method of preparing tubes of the same digestibility is to be preferred. He proceeds as in the original preparation of the tubes except that as the water cools from 90° to 80° C. a single tube containing the new egg white is dropped in at each degree change of temperature, that is, at 90°, 89°, etc. Pieces of each of these tubes as well as of the original standard tubes are then allowed to digest simultaneously in portions of the same gastric juice. One of these tubes should show a digestibility equal to that of the standard tubes. For example, the tube coagulated at 88° C. may show the proper digestibility. Then the new series of tubes should be made in the same manner as this one; that is, introduced at 88° C. The tubes thus prepared should be again checked up with the standard to see that no mistake has been made.

DETERMINATION OF TRYPTIC ACTIVITY

Principles.—Trypsin is not secreted by the stomach but occurs in the pancreatic juice. It may be found, however, in the stomach contents because of regurgitation of duodenal contents through the pylorus. *Since it is destroyed by the pepsin-hydrochloric acid of the stomach, the determination must be made immediately after securing gastric juice, especially in cases of high acidity.*

Procedure (Spencer)—1. Prepare five reagent tubes; more if desired.

2. To tubes 1 and 2 add 0.5 c.c. of gastric contents (filter if cloudy).

3. To tubes 2, 3, 4 and 5 add 0.5 c.c. of distilled water.

4. From tube 2 remove 0.5 c.c. of its mixed contents and add to tube 3. Mix thoroughly and add 0.5 c.c. from tube 3 to tube 4. Repeat for tube 5.

5. This gives dilutions of gastric contents of 1, 1:2, 1:4, 1:8, and 1:16.

6. To each tube add 1 drop of phenolphthalein solution (phenolphthalein, 1 gram; 95 per cent alcohol, 100 c.c.); then add drop by drop a 2 per cent sodium carbonate solution until a light pink color is produced.

7. To tubes 1, 2, 3 and 4 add 0.5 c.c. of casein solution. Tube 5 must receive 1 c.c. of casein solution, since it contains 1 c.c. of the diluted gastric contents. For the casein solution, dissolve 0.4 gram of casein in 40 c.c. of N/10 sodium hydroxide. Add 130 c.c. of distilled water, then 30 c.c. of N/10 hydrochloric acid. This leaves the solution alkaline to the extent of 10 c.c. of N/10 sodium hydroxide, minus about 3 c.c. neutralized by the casein.

8. Incubate for five hours at 40° C.

9. Precipitate the undigested casein by dropwise addition of a solution of the following composition: glacial acetic acid, 1 c.c.; 95 per cent alcohol, 50 c.c.;

distilled water, 50 c.c. The tubes in which digestion has been complete remain clear; others become turbid.

10. The tryptic values are expressed in terms of dilution. Thus, complete digestion in tube 3 (a dilution of 1:4) shows four times the tryptic power of undiluted gastric juice which is taken as a standard as 1; therefore, its tryptic value is 4.

11. Controls of boiled gastric contents plus casein solution, and of distilled water plus casein solution, treated as above stated, must show no digestion, and become turbid on addition of the precipitating solution.

DETECTION OF RENNIN

Principle.—Rennin is an enzyme capable of coagulating the protein of milk. Fresh milk is used, therefore, as the reagent.

Lee's Test.—1. Place 5 or 10 c.c. of fresh milk in a test tube.

2. Add 5 drops of gastric juice.

3. Place in the incubator for 15 to 20 minutes.

4. If rennin is present, coagulation will occur. In this test it is sometimes difficult to tell whether the rennin or the acid in the gastric juice caused the coagulation; however, rennin is practically always present where there is hydrochloric acid in the stomach and the test is only of value in those cases in which there is no hydrochloric acid, to determine the presence or absence of a true achylia.

Riegel's Test.—1. Place 5 c.c. of fresh milk in a test tube.

2. Add 5 c.c. of gastric juice neutralized with N/100 sodium hydroxide (phenolphthalein as indicator).

3. Place in a water bath at 40° C.

4. If rennin is present in normal amount, coagulation will occur in ten to fifteen minutes.

5. Delayed coagulation indicates a less amount.

MICROSCOPIC EXAMINATION

Procedure.—1. Place a small drop of gastric residuum on a slide and cover with a cover glass.

2. Examine with low and high objectives with the light reduced.

3. Mix a drop of fluid with a drop of sudan III on a slide and cover with cover glass. Neutral fat globules will be yellow or red.

4. Mix a drop of fluid with a drop of Lugol's solution on a slide and cover with cover glass. Starch granules will be blue or blue black.

5. Make a thin film on a slide, fix with heat and stain with dilute carbolfuchsin; dry. Examine with oil-immersion objective for Boas-Oppler bacillus (*Lactobacillus boas-oppleri*), staphylococci, yeast, sarcina, and other microorganisms. Note the organisms present and whether in small or large numbers, singly, in groups or colonies (masses).

6. With the above preparations examine for the following:

1. *Red blood corpuscles* found in small numbers cannot be considered of any pathological significance. Trauma from passing the tube and aspirating the stomach

contents will frequently give rise to their appearance. The consistent finding of large numbers in the absence of considerable trauma may be indicative of ulcer, cancer or erosion.

2. *Leukocytes* are frequently found in the gastric residuum. They are usually partially digested by the gastric juice. They may be indicative of disease from the nose or throat. If so, they are usually associated with other products of inflammation from that region which can easily be identified, e.g., mucus and exfoliating epithelium. If these cells are deeply bile-stained and associated with a bilious residuum and other inflammatory elements also bile-stained (e.g., columnar epithelium, mucus, bacteria, etc.) it is very suggestive of biliary tract
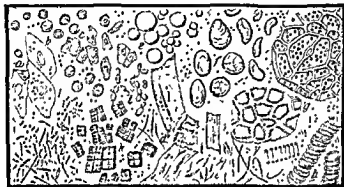
FIG. 128.—MICROSCOPY OF STOMACH CONTENTS

a, squamous epithelial cells from esophagus and mouth; *b*, leukocytes; *c*, cylindric epithelial cells; *d*, muscle-fibers; *e*, fat-droplets and fat-crystals; *f*, starch granules; *g*, chlorophyl containing vegetable matters; *h*, vegetable spirals; *i*, bacteria; *k*, sarcinae; *l*, yeast cells (Jacob). (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

disease. The presence of large quantities of pus cells with gastric exfoliative products, mucus and bacteria, would suggest gastritis of the infective type (Fig. 128).

3. *Mucus* in small amount is practically always found in the gastric residuum. A differentiation between mouth and stomach mucus can often be made by the macroscopic examination. Gastric mucus is usually present as small flocculations which under the microscope look like spherical snail-like bodies. It has a fibrillary appearance and has cellular bodies in its substance. If present in large quantities it is indicative of catarrhal gastritis. Mucus is dissolved by alkalis but not by acetic acid. Mucus from the biliary tract should be easy of identification. It occurs in wavy semi-spirals and is rather dense in appearance and stained a deep yellow or greenish yellow if it has resided in the stomach very long. The frequent finding of bile-stained mucus in the fasting stomach is suggestive of biliary tract disease. Mucus from the nose is easily recognized by its peculiar tenacity and its staining with pigment or its grayish appearance.

4. *Epithelium* is practically always present in the residuum (Fig. 128). The usual cell found is the squamous type. It has no special significance and is derived from the mouth, pharynx and esophagus. It is swallowed with mucus during the passage of the tube. Cellular elements from the stomach mucosa are of the columnar variety. They are rarely seen in the normal residuum. Cells are quickly digested in the stomach and unless they are present in considerable numbers they will not be found. In gastritis cellular elements from the gastric tubules are often found. The acid or parietal cells are probably the more easily recognized of the tubule cells. They are about midway in size between a leukocyte and a squamous cell. With eosin and hematoxylin stain the acid cells are stained red and the central or peptic cells blue. The peptic cells take the stain very poorly and are very difficult to recognize, there being nothing but the nuclei remaining. They are much smaller than the acid cells, being a little larger than a leukocyte. They have a long oval nucleus often with just a shred of protoplasm attached. The acid cells, on the other hand, have a very distinct nucleus and the entire protoplasm stains with a fine stippling of the granules. In chronic catarrhal gastritis exfoliating elements from the gastric mucosa may be found, even without a severe grade of atrophy being present. The association of these cellular elements from the gastric mucosa with colonies or groups of pathogenic organisms is indicative of an infective type of gastritis. The frequent presence of deeply bile-stained columnar epithelium in the fasting stomach associated with bile is suggestive of gall tract disease.

FIG. 129.—OPPLER-BOAS BACILLI

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

5. *Bacteria* in the gastric residuum are rarely of importance. In the ordinary tube examination a few bacteria will usually be found. However, they will be associated with oral or nasal epithelium or mucus. In other words, they have been swallowed during the passage of the tube. The presence of bacteria, particularly of the pyogenic variety, occurring in colonies or masses and intimately associated with exfoliating epithelium from the stomach, usually means an infective type of gastritis. The presence of masses of deeply bile-stained organisms, on the other hand, may indicate biliary tract disease.

The finding of the Boas-Oppler bacillus in the stomach is significant of gastric stagnation. In a great majority of the early cases of cancer the Boas-Oppler bacilli will not be found. It is not until the hydrochloric acid starts to be reduced and obstruction has occurred that one can expect to find this organism (Fig. 129).

6. *Tissue fragments* are occasionally found in the gastric residuum. Rarely in cases of cancer, small fragments of mucosa may be found showing carcinomatous infiltration and probably more often areas of necrotic tissue containing leukocytes and bacteria. In achylia gastrica and in atrophic gastritis fragments may be re-

covered showing a great diminution in the number of gastric tubules or a complete absence of tubules. A small round cell infiltration of the fragment may be noted in cases of gastritis. Small fragments of gastric mucosa may be found in peptic ulcer. This rarely happens, however, as the hyperacid juice, commonly found in this condition, soon digests any protein matter present. The finding of fragments of mucous membrane showing a hyperplasia of the glandular elements particularly of the base of the gland, would suggest a diagnosis of hypertrophic glandular gastritis. Einhorn describes the recovery of hemorrhagic flakes of gastric mucosa in the fasting residuum which he ascribes to "gastric erosions."

7. *Starch granules* are usually present and easily recognized by their concentric striations. When undigested, they stain blue with Lugol's solution; when partially digested, a reddish color due to erythrodextrin. Fat may be present, as likewise other particles of partially digested food such as muscle fibers and vegetable cells. Various crystals may be found, especially of fats, but they possess no particular significance. Animal parasites or ova may be observed (Fig. 128).

CHAPTER X

METHODS FOR THE COLLECTION AND EXAMINATION OF BILE AND DUODENAL CONTENTS BY DUODENO-BILIARY DRAINAGE

METHOD FOR COLLECTION

Duodenal contents are aspirated by syphonage (Fig. 130) through a swallowed flexible rubber tube fitted with a perforated tip by the method of Lyon. The flow of bile is stimulated either by injecting magnesium sulphate, which produces a localized relaxation (transient ileus) of the duodenal region, opening the sphincter of Oddi, or by the use of physiological cholagogic and choleretic agents, such as olive oil, peptone, or oleic acid.

Materials Required.—1. The Lyon tube is 130 cm. in length, with a pear-shaped metallic tip having an elongated grooved shank securing it to the tube without tying by thread, thereby minimizing trauma. This tube has two marks equidistant from the ends, and 20 cm. apart. The single mark at 55 cm. from the tip represents the average distance from the lips to the greater curvature of the stomach. and the double mark at 75 cm. to a position approximately at the level of the ampulla of Vater. To the outer end is attached a glass observation cannula or window. which. in turn, connects a larger size tubing. 30 to 50 cm. in length. Because the marks on this tube are equidistant from the ends, the tube may be reversed when wear and tear begin to appear on the swallowed portion. These tubes are also equipped with an adjustable rubber collar at the duodenal mark, which serves to record the variations in length effective for individual patients, and to enable the patient to feel its contact on the lips without difficulty.

Other useful tubes are those of Rehfuss, Twiss, Levin and Jutte (well adapted for use in infants).

2. Each patient is also provided with a tray containing a one ounce capacity Asepto bulb syringe, a kidney basin, a clamp, a percolator or funnel, two 250 c.c. graduates. and 125 c.c. graduate, and three or more 250 c.c. bottles fitted with perforated rubber stoppers with glass tubing inserted for the collection of bile.

3. *Stimulants for Bile Flow.*—(a) Saturated solution of magnesium sulphate, diluted with two volumes of sterile water to make a 33% volumetric solution. With this the patient is stimulated one or more times, depending upon the amount of magnesium sulphate solution that has been retained. The following fractional dose is advised: first stimulation of 45 c.c., recovering as much as possible; second stimulation of 30 c.c.; third stimulation 30 c.c. Care should be taken that not more than 90 c.c. of the 33 per cent solution (equivalent to one ounce of the saturated

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solution) is retained at any one treatment, because of the danger of a severe adynamic ileus of the upper bowel.

(b) Fifty to 100 c.c. of a 5% sterile solution of peptone (boiled and filtered).

(c) Fifteen to 30 c.c. of olive oil warmed to body temperature. Adding 15 c.c. of hot water facilitates its delivery through the tube. Since the oil rises to the top of the specimens and does not dilute the flow of bile, it affords the most satisfactory results when quantitative chemical analyses are to be carried out.

FIG. 130.—PASSING THE GASTRODUODENAL TUBE (Lyon)

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger.)

Olive oil frequently produces a more prompt flow of B-bile than occurs with magnesium sulphate, and occasionally will cause such a flow after previous drainages with the magnesium salt have failed to do so. The chief objection to the use of olive oil, however, is that it interferes with proper microscopy, especially at the hands of a beginner.

(d) Five to 10 c.c. of oleic acid, chemically pure, in 15 to 30 c.c. of water.

Procedure.—1. *Preparation of the Patient.—* The most satisfactory time for doing a diagnostic drainage is in the morning on a fasting stomach. In preparation, the patient may be instructed to eat a meat sandwich, 20 raisins, and a glass of milk or water at 9:00 P.M., the night before, as an optional motor test meal. The

reason for this test meal is to introduce easily recognized food material so that if stasis occurs, it may be detected in the aspirates the following morning.

After a twelve-hour fast, drainage is then performed the following morning at nine o'clock. Brushing of the teeth that morning should be omitted to prevent the swallowing of blood from bleeding gums. A few moments of simple explanation as to the procedure generally gains the confidence of the patient. For the beginning of the drainage, the patient, if not too ill, sits on a chair, having removed any dentures or tight clothing.

2. *Intubation.*—(a) In passing the tube, stand a little to the right of the patient, facing him with the tip of the tube in the right hand (Fig. 130), and explain that when the tip is placed in the back of the mouth, he should alternately swallow and breathe naturally through the nose, holding the head in a natural position, until the tip has passed the glottis, after which the tube may easily be slipped down to the stomach mark in the absence of esophageal obstruction or an excessive gag reflex.

(b) The fasting gastric residuum is now extracted by gravity. Only occasionally is the syringe needed to start the flow, but always with minimum suction to avoid trauma. This residue should be described and examined, especially for free and total hydrochloric acid, occult blood, bile, mucus, and detailed microscopy.

(c) The stomach is now washed with several 250 c.c. units of sterile water at body temperature through a percolator or funnel placed about 18 inches above the patient's head. This is returned by syphonage into a graduate on the floor.

(d) Following this, 100 c.c. of sterile water may be introduced through the tube into the stomach to encourage gastric peristalsis in carrying the tip through the pyloric canal, and the tube is then clamped.

(e) The patient is instructed to lie on the bed on his right side (Fig. 131) and *slowly* swallow the tube, taking 20 minutes to get the duodenal mark on the lips. One minute for each centimeter on the Lyon tube will usually engage the tip synchronously with the frequent peristaltic waves which will carry it through the pyloric canal.

(f) The tube is then unclamped and connected to the first bottle. The first fluid to appear will be either pearly gray or yellow duodenal-pancreatic fluid. The fasting duodenum frequently already contains bile.

(g) In locating the position of the duodenal tip and assuring oneself that it is at the proper level, fluoroscopy is rarely necessary. If there exists any doubt as to the location of the tip, stethoscoping the abdomen for maximum air explosions over the stomach and duodenum with air introduced under syringe pressure through the tube will prove highly reliable. An experienced technician, however, can easily and quickly locate the position of the tip when using an Asepto bulb syringe, and, with an "educated thumb" constantly controlling the pressure on the bulb, by injecting a little water will find that if the tip is in the stomach, all fluid will return quickly; if in the pyloric canal, the water will go in very slowly and there will be no return of the fluid, but only a decided tug on the bulb; if in the duodenum, the water will enter slowly and only a portion, generally bile-stained,

will return. If the tube is not in the duodenum, it is then withdrawn well up in the stomach and slowly reswallowed. A tube buckled in the stomach or at the pyloric orifice may be partially withdrawn without any initial resistance, but if the tip is in the pylorus or duodenum a slight initial·resistance is felt. Rarely a worm tube knots itself in the stomach.

3. *Stimulation for Bile Flow.*—(a) With the tip definitely in the duodenum, bile drainage is established by aerial stimulations with one or more of the stimulants described above. These solutions should all be introduced at body temperature and the tube clamped for 2 to 5 minutes before syphonage is established.

Fig. 131.—Duodenal Lavage in Sims' Position
(From Lyon, *Non Surgical Drainage of the Gall Tract*, Lea and Febiger.)

This will begin immediately provided the tube remains filled with fluid before being clamped. A mixture of magnesium sulphate and peptone will sometimes give better results than either alone, and both are satisfactory for bile microscopy and culture.

(b) Over a three-hour drainage period the amount of bile mixture usually recovered by an *adequate drainage* will total 250 to 400 c.c., including 20 c.c. of pearly gray or yellow duodenal fluid (D-bile), 10 to 20 c.c. of golden-yellow duct bile (A-bile), 30 to 75 c.c. of dark yellow-brown, mahogany, dark green, or black gallbladder bile (B-bile), and at least 200 c.c. of golden to lemon liver bile (C-bile). The appearance of these is shown in Plate VI. Collecting bottles should be changed at intervals as the bile fractions are identified.

PLATE VI
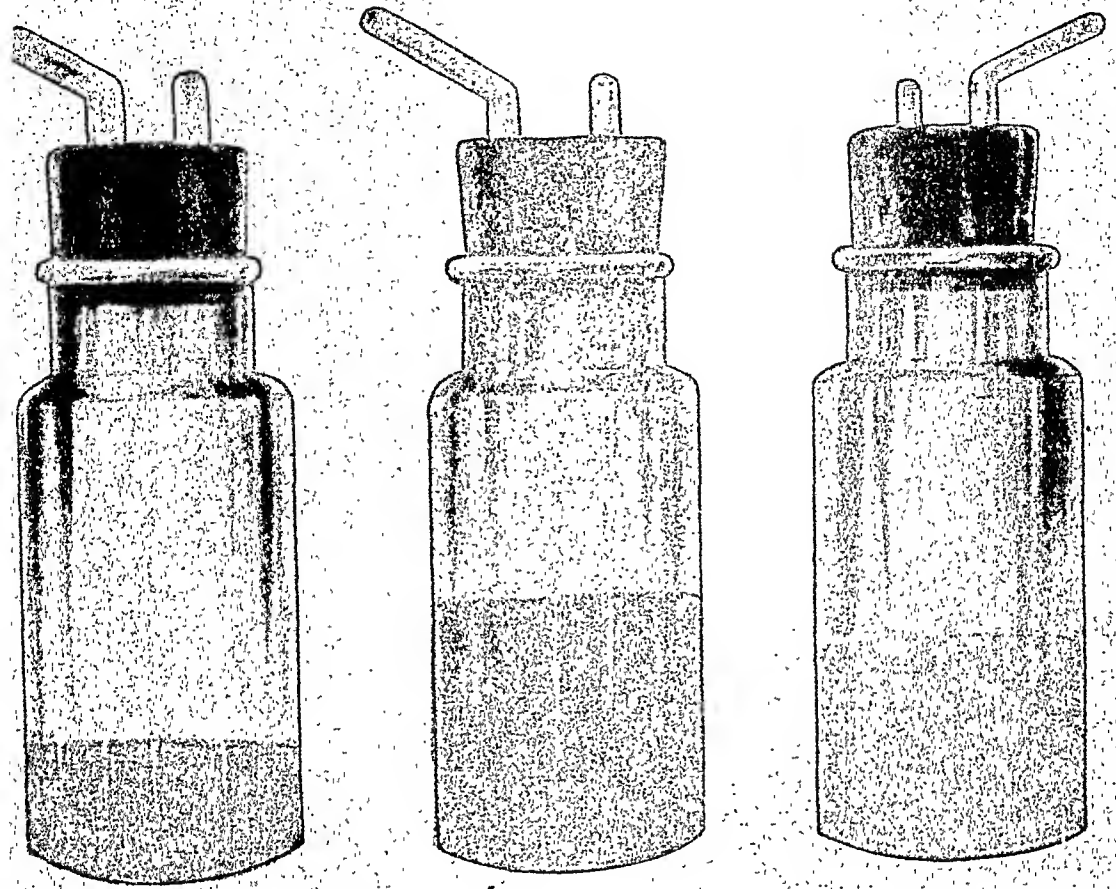
NORMAL A, B AND C FRACTIONS OF BILE.

FIG. 1.—Common duct   FIG. 2.—Gall-bladder   FIG. 3.—Liver bile
bile (chiefly).   bile (chiefly).   (pure)

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger.)

(c) *Cultures* are made as desired from any bile sample. For gallbladder culture the best material is afforded by the last of the B-bile (the dregs from the floor of the gallbladder). This is theoretically so but is practically difficult of accomplishment. Cultures are taken directly into glucose hormone bouillon in the special flasks devised by Richardson (Fig. 132). Or the bile may be collected in a sterile vial (Fig. 133) and plated in the laboratory.

(d) *Withdrawal of Tube.*—Before withdrawing the tube from the duodenum to the stomach and from the stomach to the esophagus, a little air should be blown
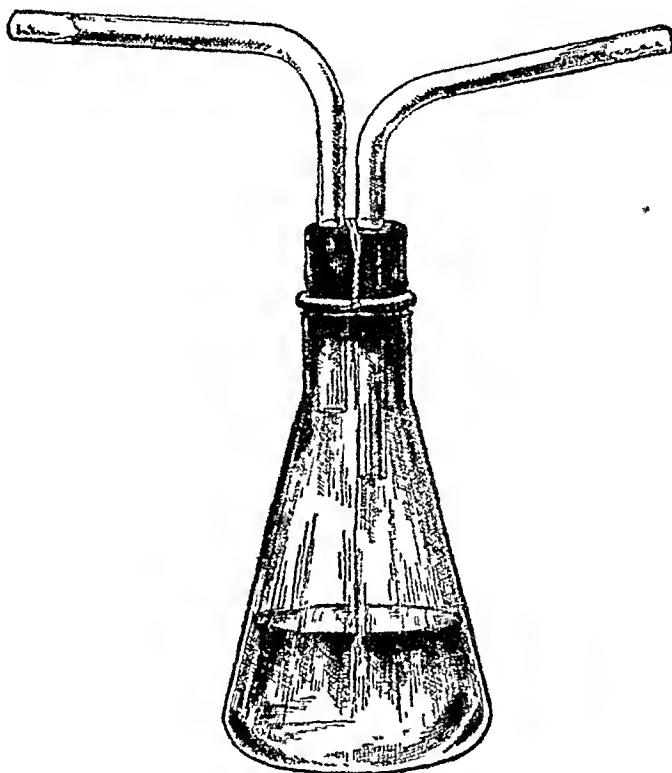
FIG. 132.—SPECIAL CULTURE FLASK

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger.)

through the tube to balloon the walls away from the metal tip to avoid scratching the mucous membrane. As the tube reaches the glottis, a swallowing movement will facilitate its withdrawal. The duodenum and stomach may be lavaged during removal as desired. A stomach wash with water during withdrawal to remove any regurgitant bile, and a mouth wash, a cup of broth, and some crackers after withdrawal add to the comfort of the patient. Tubes should be flushed out and sterilized by boiling after use. Good rubber should be used in the manufacture of these tubes.

Sources of Difficulty.—Occasionally difficulty may be experienced in getting the tip through the pylorus, or the tube may be sharply buckled in the stomach.

In such a situation withdrawal of the tip well up into the fundus is necessary prior to reswallowing. The following conditions must be considered: Faulty tech-
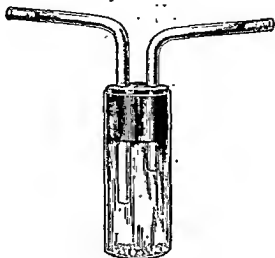
FIG. 133.—COLLECTION VIAL

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger.)

nic; pylorospasm; gastroptosis and gastric atony; organic disease of the stomach and duodenum and accidents.

MACROSCOPIC EXAMINATION

The gross description of each of the bile fractions should include:

1. *Amount.*

2. *Color.* Red and green biles are anomalous and may be obtained at times directly from the liver or gallbladder. The admixture of gastric juice will frequently cause any specimen to turn green on standing.

3. *Flocculation and precipitation.* The amount of white or bile-stained flakes suspended in or setting on the bottom of the receiving bottle in *uncentrifuged* specimens should be recorded as to degree, as follows:

$$+ = \text{occasional floccule (normal).}$$
$$++ \text{ to } ++++ = \text{pathological}$$

4. *Blood* (pure blood rarely observed).

5. *Red mucous flecks* (often associated with gastric or duodenal ulcer).

6. *Viscosity* (normally slightly viscous, due to mucin).

As a rule it may be said that normal duct, gallbladder, and liver biles will be transparent and free from turbidity, except as occasional zones of turbidity, like an egg yolk emulsion, form in the drainage bottles as spurts of acid gastric juice (physiological action of the vagus) blend with the bile. This turbidity, caused by the precipitation of bile acids, will clear up on shaking the specimen if the pH
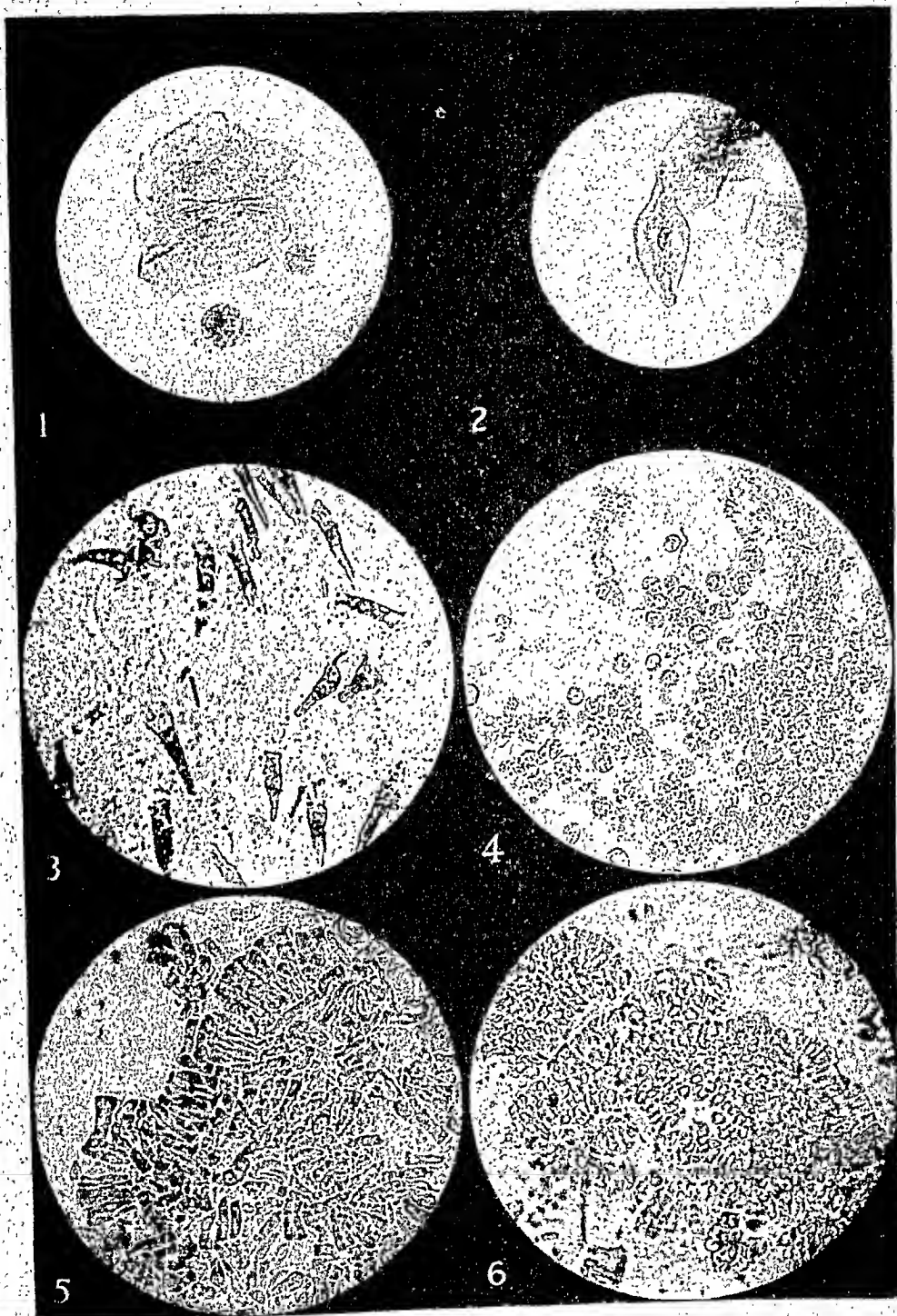
FIG. 134.—MICROSCOPY OF BILE

1, stratified pavement epithelial cells from buccal membrane of normal mouth, ×350; 2, isolated esophageal epithelial cells, ×380; 3, gastric cells with conspicuous degenerative changes, ×360; 4, duodenal cells, oval or cuboidal. Most common type in "wet" preparations, ×380; 5, gallbladder cells, bile stained, ×380; 6, gallbladder cells obtained on biliary drainage, ×380. (From Lyon, *Atlas of Biliary Drainage Microscopy*.)

of the duodenal bile mixture is sufficiently alkaline. Otherwise turbid biles are to be construed as pathological, especially when they contain much flocculent debris or sediment abundant in exfoliated epithelial cells, pus cells, bacteria, mucus, crystalline elements, or oleaginous substance.

Not infrequently in the presence of a non-functioning gallbladder, liver bile will be darker and more concentrated than normal. This concentration of bile may readily be ascertained by determining its bilirubin content, especially if olive oil has been used as the stimulant. The normal bilirubin content of liver bile, obtained by the use of olive oil, ranges from 2 to 10 mgm. per 100 c.c. in the duodenal fluid, whereas the bilirubin content of abnormal liver bile ranges from 11 to
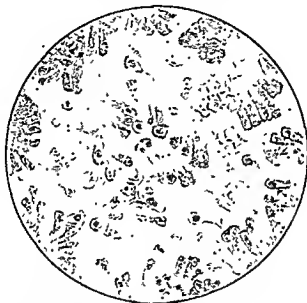
Fig. 135.—Short Columnar Bile Stained Epithelium

(From Lyon, *Non Surgical Drainage of the Gall Tract*, Lea and Febiger.)

40 mgm. per 100 c.c. when obtained under similar conditions. The "B" fraction, which is a mixture of gallbladder and liver bile, should exhibit an appreciable elevation in bilirubin content (15 to 30 or more mgm. per 100 c.c.) if the gallbladder is functioning properly as a concentrating viscus. For a method for determining the bilirubin quantitatively see Elton and Deutsch, *Archives of Pathology*, 1933, 15: 818

MICROSCOPIC EXAMINATION

1. Do not centrifuge the specimens.

2. Fish for desired floccules and sediments with a pipet and prepare wet preparations.
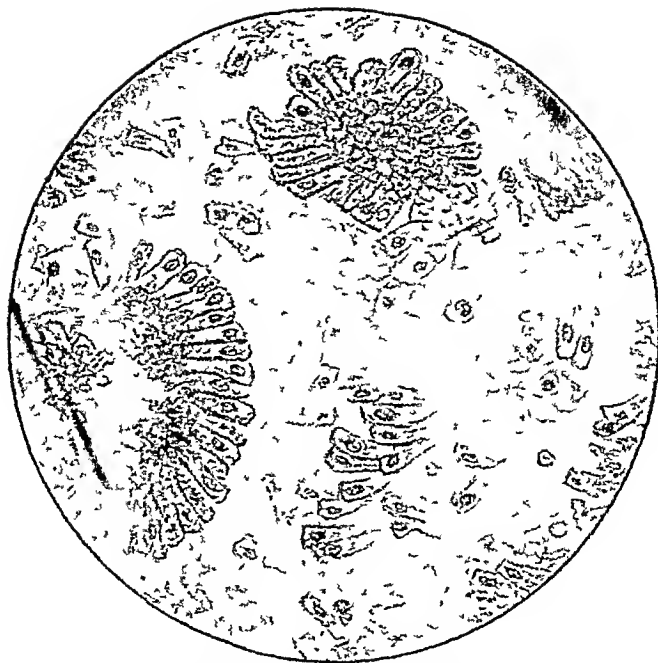
FIG. 136.—TALL COLUMNAR BILE-STAINED EPITHELIUM

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger.)
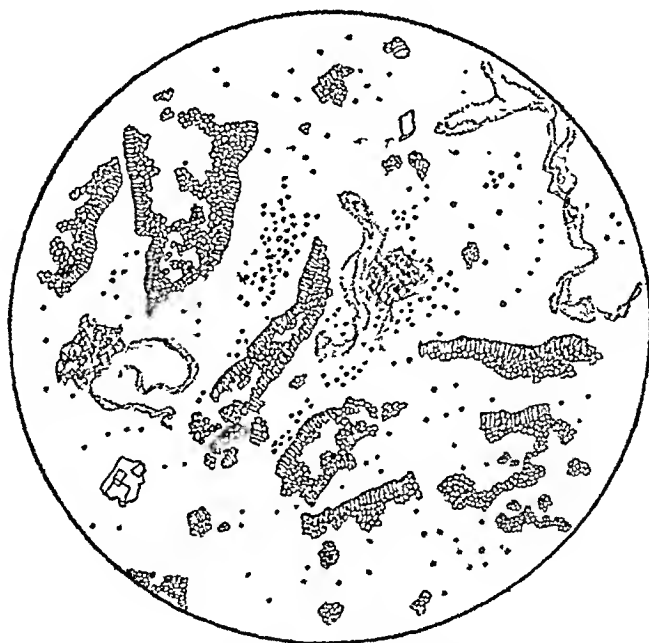
FIG. 137.—DEGENERATED BILE-STAINED COLUMNAR EPITHELIUM

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger.)
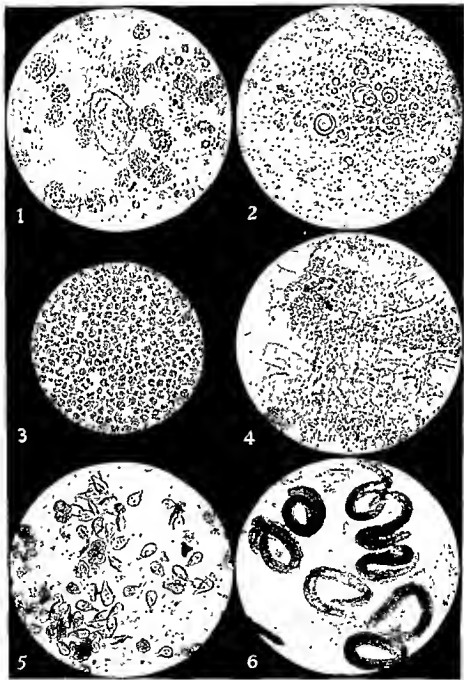
FIG. 138.—MICROSCOPY OF BILE

1, salivary corpuscles, oval or round cells containing irregular sized granules, ×380; 2, myelin threads from gastric tubules, rolled into small snail like bodies by gastric peristalsis, ×380; 3, pus cells with intact protoplasm, ×380; 4, yeast, leptothrix and mixed bacteria, ×250; 5, vegetative forms of Giardia (Lamblia) ×460; 6, larvae of Strongyloides stercoralis, Lugol's stain, ×360. (From Lyon, *Atlas of Biliary Drainage Microscopy*.)

3. Examine fresh unstained wet preparations under both low and high dry magnification.

4. Examine at least three preparations from each specimen showing flocculation.

5. Examine for the following (see Table I):

> (a) Epithelial cells which may be present from the mouth, esophagus, stomach, duodenum and gallbladder (Figs. 134, 135, 136 and 137).
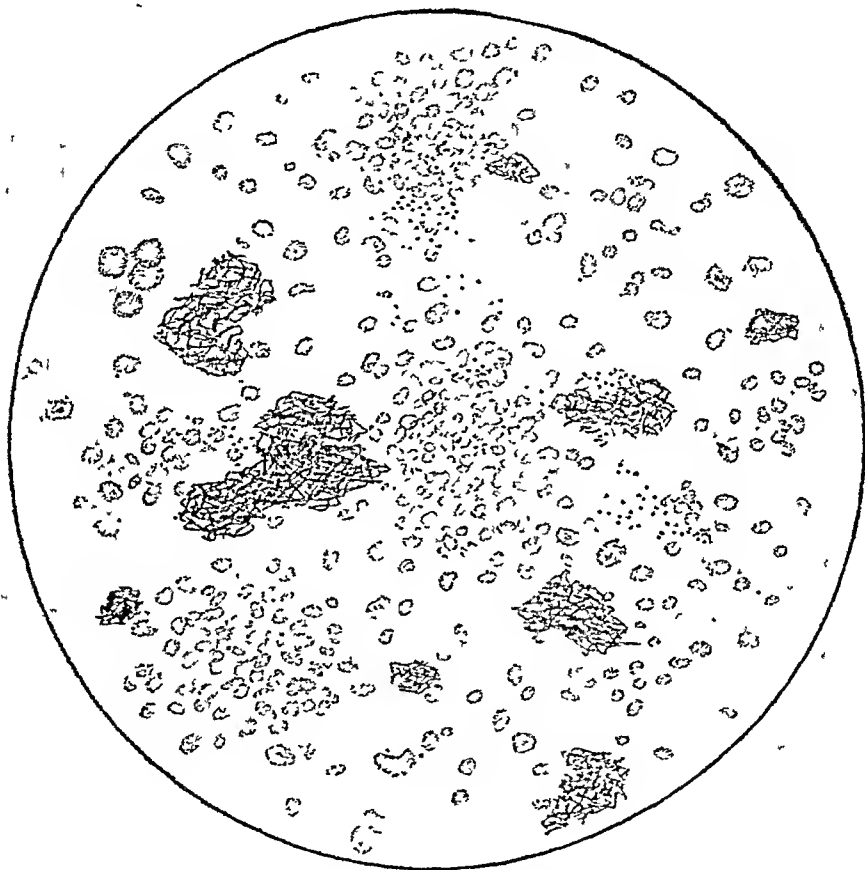
Fig. 139.—Practically Pure Pus with Occasional Necrotic Epithelial Cell and Masses of Bile Pigment

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger.)

> (b) Salivary corpuscles (Figs. 138 and 139).
> (c) Myelin threads (Fig. 138).
> (d) Pus (Figs. 138 and 139).
> (e) Micro-organisms and parasites (Figs. 138 and 140).
> (f) Mucus (Fig. 141).
> (g) Oleaginous substances (Fig. 141).
> (h) Crystals (Figs. 140 and 142).
> (i) Food (Fig. 141).

Fig. 140.—Bacterial Colony from Duodenum
(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger.)

DETECTION OF TRYPSIN, AMYLASE AND LIPASE IN DUODENAL CONTENTS

Einhorn's Method.—1. Place sufficient bile in test tube to make about 3 inches deep.

2. Place in the bile the following tubes (file off the ends, avoiding dried out ends):

> (a) Hemoglobin tube
> (b) Starch tube
> (c) Olive oil tube

PREPARATION OF HEMOGLOBIN TUBES

Hemoglobin powder	1.0 gm.
Agar powder	2.5 gm.
Water (distilled) q.s.	100.0 c.c.

Rub hemoglobin powder with 10 c.c. of water until a smooth paste is obtained; then add the agar and water. Heat and fill capillary tubes as directed for starch tubes.

PREPARATION OF STARCH TUBES

Agar powder	2.5 gm.
Starch	5.0 gm.
Water (distilled)	100.0 c.c.
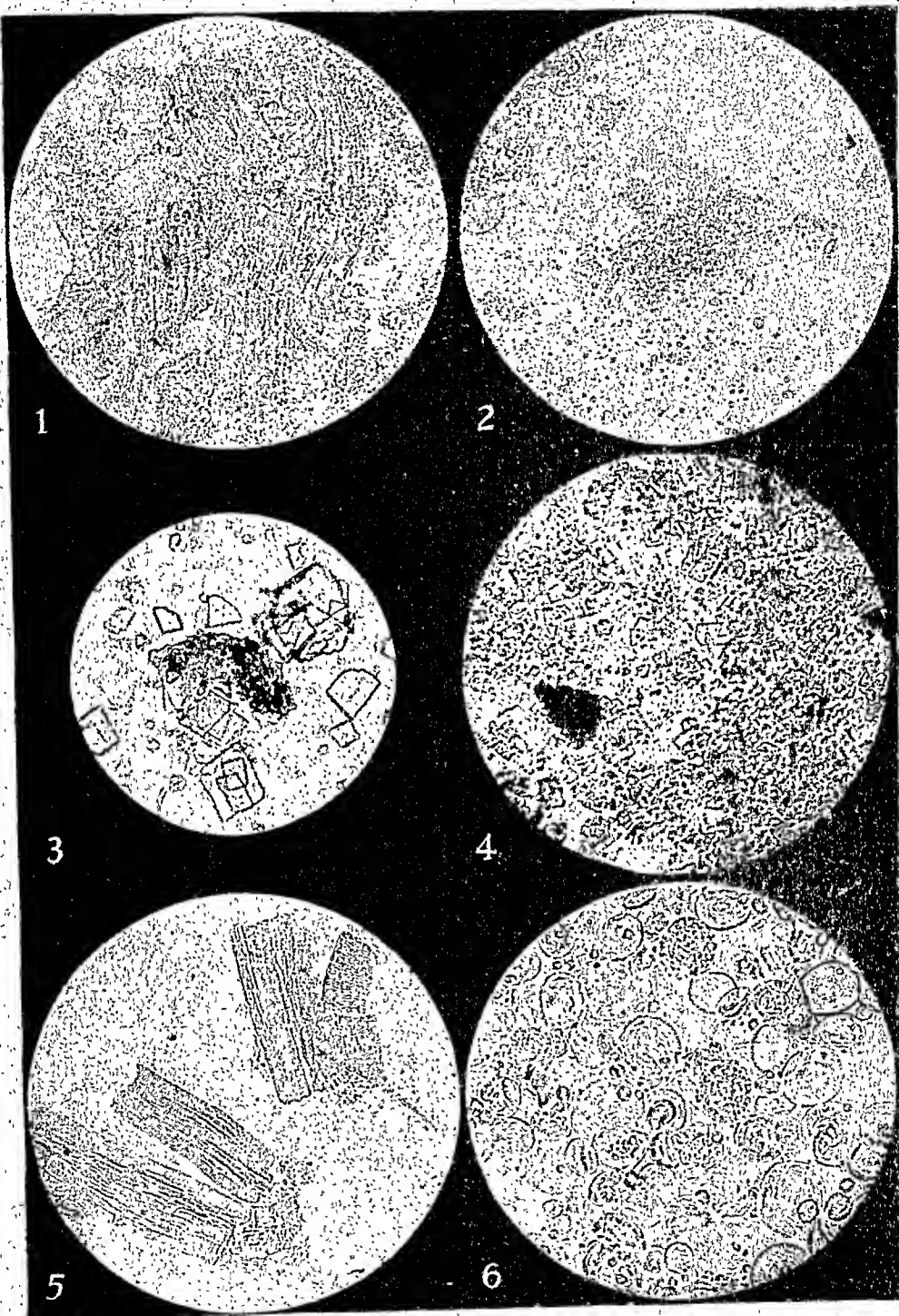
FIG. 141.—MICROSCOPY OF BILE

1, mucus ribbons or bands from duodenal zone in sheets, ×180; 2, grade one oleaginous degeneration, ×380; 3, combination of cholesterin and calcium bilirubinate crystals, ×360; 4, mixture of calcium, cholesterin and bile pigment crystals; 5, striated meat fiber, ×200; 6, starch cells from bread, ×460. (From Lyon, *Atlas of Biliary Drainage Microscopy*.)

Rub starch and agar in a mortar with sufficient water to make smooth paste, then add balance of the water.

Place in a flask and heat to boiling point.
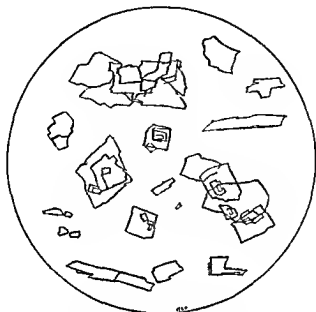
FIG. 142.—CHOLESTERIN CRYSTALS

(From Lyon, *Non-Surgical Drainage of the Gall Tract,* Lea and Febiger.)

Draw by suction while still hot into warm capillary glass tubing (inside diameter 1.5 millimeters).

After cooling cut into small pieces (3 centimeter lengths) and seal the ends with paraffin.

PREPARATION OF OLIVE OIL TUBES

Olive oil .... 25 c.c.
Agar powder .... 2 gm.
Nile blue sulphate (aq. sol. 1:2000) q.s. .. 100 c.c.

Rub olive oil and agar together to make thin paste and then add Nile blue solution to make 100 c.c. Heat to boiling; fill in capillary tubes as directed for starch tubes.

3. Place in the incubator at 37° C. for 24 hours.

4. Examine the two ends of the hemoglobin tube for clear, digested area; measure each with millimeter rule; take the average and report in numbers of millimeters. The digestion is due to the presence of trypsin.

5. Examination of starch tube. Push the contents of tube out on a slide and cover with Lugol's solution; measure the part which does not turn blue as above

TABLE I[1]

Object	Description	Cause of Excess	Comment
Oral mucous membrane	Stratified squamous epithelium	Stomatitis	May be accompanied by pus cells
Salivary corpuscles	4 to 5 times size of leukocyte: twice size of duodenal cell: oval or round, containing granules of irregular sizes		Rarely reach duodenum. May liberate granules as irregular, dense translucent gray bodies. probably lecithin, mucin, or myelin. Lecithin-like bodies at times found in bile.
Esophageal cells	Stratified squamous epithelium, with tendency to more narrow diamond-shaped pattern in deeper layers. More variation in size and shape of nucleus.	Simple esophagitis due to mucosal irritants: cardiospasm: cancer of esophagus: diverticulum.	Patients present deglutition difficulties
Myelin threads	Small snail-like bodies	Hyperperistalsis: high gastric acidity	Probably pressed out of gastric tubules: may be mucin, or derived from saliva
Pus cells	Nucleus prominent when cytoplasm digested by normal or increased HCl: not digested if hypochlorhydria exists. Bile stained pus cell suggests biliary tract inflammation	Inflammation in zone where found or in adjacent zone above	Nuclei resist gastric digestion, but are readily attacked by pancreatic trypsin
Gastric cells	(a) Tall columnar cell predominates: goblets with biforked tails: usually not bile stained (b) Columnar, but scanty, degenerated, thick-bodied, and hyalin in appearance	(a) Irritants (b) Usually in chronic progressive atrophic gastritis	Oval and cuboidal cells of gastric epithelium do not exfoliate to appreciable degree
Duodenal cells	(a) Oval or cuboidal cell with single nucleus: often twice size of leukocyte: pearly gray unless bile-stained when devitalized (b) Same, with degenerative changes of hyaline and amyloid character, and increased in thickness and density: vacuolization and inclusion bodies in nucleus and cytoplasm (c) Atypical cell: larger and resembles resting amoeba, with granules bunched in cell, leaving small zone of clear cytoplasm	(a) Duodenitis (b) Chronic duodenal disease (c) Deep-seated duodenal ulcer: cancer of pancreas eroding through duodenal wall	(a) Exact origin and nature still obscure, but represents the typical cell of the duodenum (c) Phagocytic cells? Possibly derived from pancreas

[1] Compiled from Lyon, *Atlas of Biliary Drainage Microscopy.*

Object	Description	Cause of Excess	Comment
Gallbladder cells	Single layers of simple tall columnar epithelium below which lie collections of polygonal cells arranged in rows, fans, or rosettes. Bile-stained if devitalized	Cholecystitis: antedates all other diagnostic signs	"Polygonal" cells may sometimes be columnar cells viewed on end. Short columnar epithelium may be derived from duct epithelium
Micro-organisms	(a) Bile-stained bacterial colonies (b) Diffuse bacteria (c) Leptothrix-fungus-yeast group (d) Algae or diatoms	(a) Infection of biliary tract (c) Secondary invaders (d) Seasonable, from water supply	(a) must be proved viable by culture
Mucus	(a) Duodenal mucus appears as well defined ribbons or bands: not necessarily bile-stained: may be tinged rusty red from hemoglobin products (b) Twisted or spiral mucus: invariably bile-stained: often dense and occasionally encrusted with granular deposits of brilliant yellow pigment or (?) bile acids (c) Grossly visible mucus plugs	(a) Catarrh. Red mucus associated with duodenitis, erosion, or ulcer (b) Catarrh of ducts, especially cystic duct (c) from ducts	(a) Duodenal cells accompanying duodenal mucus are usually bile-stained (b) Conformation of Heisterian valve would cause twisting (c) Presence may check recession of catarrhal jaundice at times
Oleaginous substance	Yellow oily fluid as droplets, pools, and lakes: may melt out from unusually dense twisted and bile-stained mucus. They appear as amorphous bright yellow or orange substance, from which pools and lakes emerge on standing	Catarrh of bile ducts, especially of cystic duct when present with twisted mucus: also occurs occasionally with cholesterosis of gallbladder	Probable fatty ester of cholesterol: may form actual cholesterol crystals. May be associated with cystic duct occlusion until expelled. Olive oil droplets are colorless under the microscope.
Crystals	(a) Cholesterol plates: flat, colorless, thin fragments with chipped edges like splintered window glass (b) Calcium bilirubinate: clusters of lustrous bright yellow, lemon, or orange finely or coarsely granular crystals: lustrous brilliancy differentiates from bile acids (c) Calcium: dirty grayish white, dense, thick, chunky	All crystals imply formed or forming calculi. They may also be a component of gall sand which drains off	(a) Especially significant when the more imperfect in form, or when associated with calcium bilirubinate (b) The most gritty of all the biliary crystals under the cover slip (c) Like other crystals, must be differentiated from vari-

Object	Description	Cause of Excess	Comment
	and irregular in size and shape		ous artefacts, such as dust particles, sterilizer rust, or rubber flecks from old tubes
	(*d*) Bile pigment: reddish amber to brown or black, with occasional reddish or yellow tints on thinned edge		
	(*e*) Tyrosine: long slender needles		(*e*) Should be differentiated from fatty acid crystals
Parasites	(*a*) Giardia: usually attached to ribbons of duodenal mucus or to duodenal cells by their sucker - like peristomes: dumbbell-shaped nucleus resembles pair of eyes: pear-shaped with eight flagellae arranged in pairs: average 12-20 by 8-12 micra: resemble bowl of spoon when viewed on side: more refractile than cells: tumble and gyrate when motile: stunned by action of magnesium sulphate	(*a*) Infestation: potential pathogens	(*a*) Fairly common: transmitted by cysts from carriers handling fruits, vegetables, or water supply. Often found in enormous numbers. Resistant to treatment.
	(*b*) Strongyloides stercoralis larvae: actively motile	(*b*) Infestation	(*b*) Relatively rare except in tropics
	(*c*) Various rarer parasites (entameba histolytica, clonorchis sinensis, distoma hepaticum, chilomastix mesnili, ankylostoma duodenale, trichomonas and cercomonas hominis. ascaris lumbricoides)	(*c*) Infestation	(*c*) Ascaris worms may be vomited as a result of stimulation of gag reflex
Food	(*a*) Starch cells from bread: oval and spherical bodies: some closely resemble tenia ova (*b*) Canned peas: might be confused with ova of diphyllobothrium latum (*c*) Lima beans: might be mistaken for ascaris ova	Excess indicates stasis and desirability of x-ray studies when found in fasting stomach or duodenum	For further description of microscopic food particles consult Lyon's *Atlas*.

and report in millimeters. The digested portion fails to give the starch reaction with iodine. The digestion is due to the presence of amylase.

6. Examine the ends of the olive oil tube and measure the portion which has turned violet. The Nile blue in the digested portion turns violet. Report in number of millimeters as above. The digestion is due to the presence of lipase.

BACTERIOLOGICAL EXAMINATION

1. Smears of unstained and stained material may be examined for preliminary data as described above.

2. Cultures should not be made if there is excessive retching and gagging because of the chances of contamination.

3. Cultures are indicated (a) when large numbers of organisms are seen in smears; (b) in cases requiring a careful search for foci of infection; (c) in cases of obvious gallbladder infection in which vaccine therapy is to be tried.

4. The technic is described and bacteriological findings are given in Chapter XIX.

CHAPTER XI

METHODS FOR CONDUCTING LIVER FUNCTION TESTS

Principles.—Although jaundice, defined as a quantitative or qualitative abnormality in serum bilirubin, is a cardinal sign of a disturbance in liver function, the multiplicity of functions carried on by this organ necessitates a corresponding multiplicity of tests for the detection of specific functional aberrations. even when jaundice itself is readily demonstrable.

Among the metabolic processes conducted wholly or largely by the liver, and the more important function tests based upon them, the following may be tabulated:

FUNCTIONS	FUNCTION TESTS
1. Deaminization and the formation of urea.	1. Amino-acid tolerance: blood urea determination: Million's test for tyrosinuria: blood ammonia determination.
2. Uric acid destruction.	2. Blood uric acid determination.
3. Excretion of bilirubin.	• 3. Serum bilirubin tests: bilirubin tolerance: icterus index.
4. Glycogen storage.	4. Galactose tolerance: levulose tolerance: blood sugar determination: Burger test.
5. Excretion of cholesterol and formation of cholesterol ester.	• 5. Blood cholesterol and cholesterol ester determinations:
6. Detoxification.	6. Santonin test: hippuric acid test.
7. Selective excretion of foreign dyestuffs.	7. Rose bengal: bromsulphalein: azorubin-S
8. Formation of fibrinogen.	8. Blood fibrinogen determination
9. Formation of serum protein.	9. Albumin-globulin ratio: Takata-Ara.
10. Urobilinogen control.	10. Urobilinogen tests on urine and feces.
11. Contribution of a factor essential for erythrocyte regeneration.	11. Red cell diameter.

Many of these tests are not sufficiently understood to warrant routine clinical use. Others, not listed here, are being devised in steadily increasing numbers. Correlations of these various tests have not been fully worked out at present.

METHOD FOR DETERMINING THE ICTERUS INDEX

Principle.—The icterus index is a physical measurement of the yellow color density of blood serum by colorimetric comparison with the standard 1-10,000

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potassium dichromate solution, which arbitrarily represents unity in color density.

Standard Solution.—Dissolve 1 gram of potassium dichromate in 90 c.c. of distilled water. Add 0.1 c.c. of concentrated sulphuric acid and dilute to 100 c.c. in a volumetric flask with water.

For use dilute 10 c.c. to 1000 c.c. with distilled water, making a 1 : 10,000 solution of potassium dichromate. The yellow color density of this solution represents one icterus index unit.

Procedure.—1. With a dry needle and syringe draw about 5 c.c. of blood from a vein; place the blood in a plain tube, allow clot to form, centrifuge, and remove the serum. It is essential that the serum be free of the slightest visible hemolysis and chyle (blood should be taken after period of _fasting_).

2. Dilute 1 c.c. of serum with a measured amount of physiological saline solution until its color approximates that of the standard.

3. Compare the serum in the Duboscq colorimeter with the icterus index standard solution set usually at 15 mm. When intense jaundice is present, the standard may be set at 30 mm.

4. The computation is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of serum}} \times \text{dilution} = \textit{Units of 'icterus index.}$$

As comparison is sometimes difficult in a colorimeter because of variations in shades and degrees of opalescence, the following standards may be employed:

1. Dissolve 1 gram potassium dichromate in water, add 2 drops concentrated sulphuric acid, and dilute to 100 c.c. in a volumetric flask. Into eleven dry, clean, ordinary test tubes pipet 10, 5, 3, 2, 1.5, 1.2, 1, 0.7, 0.5, 0.3 and 0.1 c.c. of the above dichromate solution, and with a 10 c.c. Mohr pipet dilute the last ten tubes to 10 c.c. with distilled water. Mix. Clean eleven small test tubes (10x100 millimeters) with cleaning solution, wash and dry. Fill above two-thirds full with above solution. Label the tubes respectively 100, 50, 30, 20, 15, 12, 10, 7, 5, 3, 1. These may be tightly stoppered or preferably closed by sealing the glass. These labeled numbers correspond to icteric index units.

Notes.—1. Normal serum has an index of 3 to 5.

2. An index of 6 to 15 is the range of latent jaundice (without clinical signs).

3. Fasting blood should be used as the lipemia after a meal interferes with the reading.

4. Low values may be found in secondary anemia.

5. Yellow pigments in the food (carrots) give a false reading and are to be avoided 24 to 48 hours before the test.

THE VAN DEN BERGH TEST FOR BILIRUBIN IN THE BLOOD

Principle.—Bilirubinate reacts with the diazonium bodies of Ehrlich's diazo reagent in an aqueous medium without alcohol to form a reddish dye, azobilirubin. Bilirubin does not give this reaction without alcohol. Van den Bergh, finding that bile gave this reaction without alcohol, applied the principle to serum.

Procedure.—1. Place 1 c.c. of serum in a graduated centrifuge tube.

2. Slant the tube to an almost horizontal plane and allow 0.5 c.c. of Ehrlich's diazo reagent to run down the wall of the tube from a pipet so that it will be overlaid on the serum.

Ehrlich's Diazo Reagent.—Freshly prepared before using by mixing the A and B solutions in the proportion of 5 c.c. of A with 0.15 c.c. of B.

SOLUTION A

Sulphanilic acid	1 gm.
Hydrochloric acid, C.P.	15 c.c.
Distilled water, to	1000 c.c.

SOLUTION B

Sodium nitrite, C.P.	0.5 gm.
Distilled water	100.0 c.c.

Solution A will keep indefinitely. Solution B should be freshly prepared every six weeks.

3. The contact zone between the serum and the reagent is examined against a good background (sky or glazed glass window) for the development of a reddish ring, which, however faint, is indicative of the positive reaction.

4. If, in very weak reactions, the presence of a ring is suspected, it will become well defined in one or two minutes if the reaction is positive.

5. If the reddish ring is demonstrable within two minutes the reaction should be recorded as positive. If not demonstrable within 2 minutes but apparent within 10 minutes, the reaction should be recorded as "positive in —— minutes."

Comments.—The use of this technic practically eliminates the former "delayed" reaction. Most positive reactions develop within one minute. The reaction is negative if no change occurs within 10 minutes. The outstanding advantage of this technic, aside from its extreme sensitivity, is that color contrasts are afforded in a single tube. The contact zone may be lowered as desired by a slight shaking of the tube, allowing further contrast between clear serum and a serum-reagent mixture.

QUANTITATIVE SERUM BILIRUBIN DETERMINATION

This test is based upon the principle that bilirubin as well as bilirubinate, will diazotize in the presence of alcohol.

It is now believed that the Van den Bergh method does not certainly differentiate between obstructive and nonobstructive jaundice and certainly does not distinguish between a primary obstructive and primary hepato-cellular jaundice. Many now believe that the promptness of the reaction—the so-called "direct reaction"—is due to free bilirubin which is almost always present in deep jaundice with high serum bilirubin readings.

Procedure.—1. After the determination of the Van den Bergh reaction as given, shake the tube containing the serum and diazo reagent and add about 3 c.c. of 95 per cent alcohol (containing a trace of ether).

2. If a definite pinkish color fails to appear within two or three minutes, and only a white turbidity occurs, the quantitative reaction may be recorded as "too low to measure" or "less than 0.25 milligrams per 100 c.c."

3. If a very definite pink or ruby red color does develop, add 1 c.c. saturated ammonium sulphate, shake gently, and centrifuge for five or ten minutes.

4. On removal of the tube from the centrifuge, three layers will be seen: a bottom layer of clear colorless ammonium sulphate, a middle layer of compact precipitated serum proteins, and an upper layer of pink or ruby red alcoholic solution of azobilirubin. Determine from the graduations on the centrifuge tube the volume in cubic centimeters of the upper colored layer, and calculate the dilution factor as follows:

$$\frac{\text{Volume of upper layer}}{\text{Volume of serum originally used}} = dilution\ factor$$

5. In the Duboscq colorimeter compare a portion of the upper layer (pipet or pour off into colorimeter cup) with the bilirubin standard solution. For weak reactions the standard may be set at 2 mm., for strong reactions, at 5 or 10 mm. The calculation is as follows:

$$\frac{\text{Reading of standard} \times \text{dilution factor}}{\text{Reading of unknown}} \bigg/ 2 = \frac{\text{milligrams of bilirubin}}{\text{per 100 c.c. of serum}}$$

Bilirubin Standard Solution.—Dissolve 2.16 grams anhydrous cobalt sulphate in distilled water and dilute to 100 c.c. or dissolve 3.92 grams of hydrated cobaltous sulphate crystals in water, add 0.5 c.c. sulphuric acid, C.P., and dilute to 100 c.c. Either of these standards will keep indefinitely in the dark, and each is the equivalent of 0.5 milligrams of bilirubin per 100 c.c., in the form of azobilirubin.

Comment.—When bilirubinate has been shown to be present by the Van den Bergh reaction, the middle layer of proteins will often be faintly pink in color, indicating a loss of pigment from the upper layer. This loss is not clinically relevant, but if one wishes to avoid it, Van den Bergh has recommended that the serum first be diluted with distilled water before the addition of the diazo reagent, in order to break up the adsorption of bilirubinate by serum proteins. This modification of the technic will increase materially the yield of bilirubin in some sera, but will not do so in any serum unless the Van den Bergh reaction has been found to be positive.

Normally, there is less than 0.25 mgm. per 100 c.c.

ROSE BENGAL TEST

Principle.—This test depends upon the specific elimination from the blood stream of the nontoxic dye. rose bengal (disodium-tetraiodo-tetrachlor-fluorescein), by the liver.

Procedure.—1. 10 c.c. of blood is withdrawn from a vein and oxalated.

2. 10 c.c. of a sterile 1 per cent solution of rose bengal (Coleman and Bell) is injected through the same needle.

3. This syringe is now discarded, and a fresh syringe, filled with normal saline, is connected to the needle, and the saline is injected slowly over a three-minute interval to keep the needle open.

4. 10 c.c. of blood is withdrawn at the end of the three-minute interval and oxalated.

5. Exactly 6 minutes later 10 c.c. of blood is withdrawn and oxalated. The patient is advised that his stools will be red and that he is to remain in a subdued light for twelve hours to avoid a skin reaction from photosensitization.

6. The blood specimens are centrifuged and the plasma removed.

7. Acetone (C.P.) is added to each plasma in the ratio of two parts of acetone to one part of plasma. The tubes are shaken well and centrifuged.

8. The acetone extract of each is poured off into clean tubes and 0.5 c.c. of a saturated solution of sodium hydroxide (50% weight to volume) is added to each acetone extract to remove fat and bile pigment. These tubes are shaken several times during the next half hour and again centrifuged. The clear top fluids are then ready for comparison, unless considerable bile pigment is left from heavily jaundiced specimens, requiring one or two more treatments with sodium hydroxide for its removal.

9. Colorimetric readings are made by comparing the second (three minute) extract with the third (nine minute) extract. If exact matching cannot be obtained, due to a deeper yellow rose color on one side, this can be corrected by holding a compensating filter of lightly picric acid stained cellophane or celluloid under the less yellow cup and an unstained disk of the same material under the other. Several such filters of different shades are of value. The calculation is as follows:

$$200 \text{ minus } \frac{200 \times Rs}{Ru} = \text{per cent liver function.}$$

Rs is the colorimeter reading of the three minute sample, and Ru is the colorimeter reading of the nine minute sample.

Photelometer readings may be made by using a Wratten No. 74 filter and the acetone extract from the first (pre-dye) sample in the middle cup, instead of water. The galvanometer readings are then transformed into readings of milligrams per 100 c.c. by comparison with a standard curve worked out by Giordano and Eager (see Table p. 238). The following calculation is then used:

$$200 \text{ minus } \frac{200 \times M2}{M1} = \text{per cent liver function.}$$

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TABLE I

Calibration Table for Rose Bengal Determinations [1]

Photelometer Readings	Rose Bengal	Photelometer Readings	Rose Bengal	Photelometer Readings	Rose Bengal
	mgm.		mgm.		mgm.
25.0	3.560	50.0	1.139	75.0	0.414
25.5	3.509	50.5	1.116	75.5	0.405
26.0	3.416	51.0	1.092	76.0	0.395
26.5	3.325	51.5	1.069	76.5	0.386
27.0	3.236	52.0	1.046	77.0	0.377
27.5	3.149	52.5	1.023	77.5	0.368
28.0	3.062	53.0	1.000	78.0	0.359
28.5	2.978	53.5	0.983	78.5	0.350
29.0	2.895	54.0	0.967	79.0	0.342
29.5	2.814	54.5	0.951	79.5	0.333
30.0	2.733	55.0	0.934	80.0	0.321
30.5	2.654	55.5	0.918	80.5	0.316
31.0	2.576	56.0	0.903	81.0	0.307
31.5	2.500	56.5	0.887	81.5	0.298
32.0	2.446	57.0	0.872	82.0	0.290
32.5	2.391	57.5	0.857	82.5	0.281
33.0	2.342	58.0	0.842	83.0	0.273
33.5	2.265	58.5	0.826	83.5	0.265
34.0	2.241	59.0	0.812	84.0	0.256
34.5	2.191	59.5	0.797	84.5	0.248
35.0	2.142	60.0	0.782	85.0	0.240
35.5	2.093	60.5	0.768	85.5	0.232
36.0	2.047	61.0	0.753	86.0	0.221
36.5	2.000	61.5	0.739	86.5	0.216
37.0	1.960	62.0	0.725	87.0	0.208
37.5	1.918	62.5	0.712	87.5	0.200
38.0	1.877	63.0	0.697	88.0	0.192
38.5	1.837	63.5	0.683	88.5	0.181
39.0	1.798	64.0	0.669	89.0	0.176
39.5	1.759	64.5	0.656	89.5	0.168
40.0	1.721	65.0	0.642	90.0	0.160
40.5	1.683	65.5	0.629	90.5	0.153
41.0	1.645	66.0	0.616	91.0	0.145
41.5	1.608	66.5	0.602	91.5	0.138
42.0	1.572	67.0	0.589	92.0	0.130
42.5	1.536	67.5	0.576	92.5	0.122
43.0	1.500	68.0	0.563	93.0	0.115
43.5	1.472	68.5	0.550	93.5	0.107
44.0	1.444	69.0	0.538	94.0	0.100
44.5	1.418	69.5	0.525	94.5	0.092
45.0	1.391	70.0	0.513	95.0	0.083
45.5	1.365	70.5	0.500	95.5	0.074
46.0	1.338	71.0	0.490	96.0	0.066
46.5	1.312	71.5	0.480	96.5	0.058
47.0	1.287	72.0	0.471	97.0	0.049
47.5	1.262	72.5	0.461	97.5	0.041
48.0	1.237	73.0	0.451	98.0	0.033
48.5	1.212	73.5	0.442	98.5	0.024
49.0	1.188	74.0	0.433	99.0	0.016
49.5	1.163	74.5	0.423	99.5	0.008

M1 is the milligram per cent concentration of the three-minute specimen, and M2 is that of the nine-minute specimen.

Interpretation.—A normal liver excretes about 50% of the injected dye in 6 minutes. Normal values for the test are 85 to 110%. The lowest values (20 to 50% function) have been found in catarrhal jaundice and cirrhosis. Values of 50 to 85% have been found in the toxemias of pregnancy, acute and subacute cholecystitis, diffuse hepatitis, and a few advanced metastatic carcinomas of the liver. The result obtained indicates the amount of functioning liver left, rather than the type of lesion that exists.

GALACTOSE TOLERANCE TEST

Principle.—Galactose, a readily assimilated simple sugar without a renal threshold, is promptly converted into glycogen by the normal liver, while the remainder of the organism is apparently unable to utilize or store it.

Procedure.—1. After an overnight fast the patient empties his bladder, this specimen being saved as a control.

2. The patient then drinks a solution of 40 grams of galactose in 500 c.c. of water flavored with lemon juice. Thereafter he may drink water freely.

3. Specimens of urine are collected hourly from the resting patient for the next five hours, and each is placed in a separate container, appropriately labeled.

4. At the conclusion of the five hours, each specimen is tested for reducing substances with Benedict's *qualitative* reagent. Those giving positive reactions are mixed, and their combined volume determined.

5. The total content of reducing substances in grams present in the combined specimens is determined by the quantitative method of Benedict.

Sources of Error.—Although the excretion of galactose in the urine of diabetic individuals is usually comparable to that of normal persons, it is advisable, due to an average increase of 40 milligrams per 100 c.c. in blood dextrose during the test, to perform fermentation tests on the urine of all patients with frank diabetes or diabetic dextrose tolerance curves, regardless of the presence or absence of glycosuria in the fasting state. In patients with thyrotoxicosis the result should also be interpreted with care.

Interpretation.—The normal fasting person will utilize nearly all of the galactose given in this test and excrete up to a total of 3 grams in the urine during the five-hour period. Strongly positive tests (excretion of 6 grams or more) should be regarded as indicative of serious involvement of the liver. A markedly decreased tolerance is frequently found in hepatocellular jaundice.

BROMSULPHALEIN TEST

Principle.—Bromsulphalein (disodium phenoltetrabromphthalein sulphonate), when injected into the blood stream, is rapidly removed by the liver and excreted in the bile.

Procedure (Rosenthal and White).—1. Weigh the patient and calculate the amount of dye required for the test, allowing 2 milligrams per kilogram of body

weight. The body weight of the patient in pounds divided by 55 will give the exact quantity in cubic centimeters of the 5% solution required.

2. Inject the required amount of the dye into an arm vein, taking 1 minute to complete the injection, with care not to infiltrate the tissues outside of the vein (a 5% solution is dispensed in ampules by Hyson, Westcott and Dunning, Baltimore).

3. Thirty minutes after injection draw 5 c.c. of blood from the opposite arm. In cases of suspected early liver disease it is also advisable to obtain an additional sample exactly 5 minutes after injection.

4. Centrifuge and separate serum without delay.

5. Divide serum into two halves in two test tubes of the same diameter as the color tubes of the colorimeter. To one add a drop of 10% sodium hydroxide: to the other add a drop of 5% hydrochloric acid.

6. Estimate the amount of dye retained by comparing with the series of standards. The tube of clear acidified serum is placed behind the standard in the comparator box and a tube of water is placed behind the unknown. (A Rosenthal colorimeter with standards may be obtained from Hyson, Westcott and Dunning, Baltimore.)

Interpretation.—Thirty minutes after injection the serum of normal individuals is entirely free from the dye or contains but a faint trace. Five minutes after injection an average of 35% (20 to 50%) is retained. Increased dye retention may be regarded as evidence of a somewhat proportionate degree of impairment of liver function.

THE TAKATA TEST

Principles.—The mechanism of the test is not entirely clear. In the course of severe hepatic disease, changes in the serum occur which permit the precipitation of mercuric oxychloride from the test reagents, which precipitation is prevented by the presence of normal serum. The exact nature of these changes in the serum are not known but may be a disturbance in the proportions of the various serum-globulin fractions.

Method.—1. In each of a row of eight test tubes, place 1 c.c. of normal saline. Add 1 c.c. of serum to the first test tube making serial dilutions of the serum by taking 1 c.c. from the first test tube and adding to the second tube and then taking 1 c.c. from the second test tube and adding it to the third, and so on to the eighth tube, from which 1 c.c. is discarded. In this way, dilutions of the serum from 1:2 to 1:256 will be obtained. It is also advisable to have a control tube of 1 c.c. of normal saline.

2. To each test tube add 0.25 c.c. of 10% sodium carbonate and mix well. Then add to each test tube 0.15 c.c. of 0.5% bichloride of mercury solution.

3. Read in one-half hour and again in 24 hours.

4. A positive reaction is said to result when there is a persistent, dense, felt-like flocculent precipitation in at least three test tubes, at least one of which should contain the serum in concentrations of 1:32 or higher. As a matter of fact, when the reaction is definitely positive, the precipitation is usually present in more of the

ubes. The exact significance of precipitation in less than three test tubes is not ully understood but such a result is probably abnormal and should be considered weakly positive one. A very fine granular precipitation is frequently seen in the igher dilutions and also in the control, but this is without significance. Very oarse flocculi that frequently form in the first one or two test tubes frequently edissolve on standing and are without significance.

5. As the test was originally done, a mixture of solutions of basic fuchsin and bichloride of mercury was used instead of the latter alone. This has been largely abandoned because the color changes that result have been found to be of no importance.

6. The Takata test is positive in a very high percentage of patients who have advanced liver disease, particularly cirrhosis.

7. The test can be carried out upon ascitic fluid with the same technique and the results obtained with ascitic fluid have the same significance as those obtained with serum.

CHAPTER XII

METHODS FOR THE EXAMINATION OF FECES

MACROSCOPIC EXAMINATION

In the complete routine examination of feces, attention should be given the following:

1. *Quantity.* The average adult stool varies from 100 to 200 grams (3 to 10 ounces) but is usually around 100 grams. It is much larger (up to 350 grams) upon a vegetable diet.

2. *Form and consistency.* (a) Hard and scybalous as in constipation; (b) soft and formed (normal); (c) mushy or liquid, as in diarrhea; (d) gaseous (fermentative); (e) flattened or ribbon-like, as in spastic colitis or obstruction, etc.

3. *Color* is greatly influenced by diet and drugs:

    (a) Light or dark brown (normal) due to hydrobilirubin

    (b) Yellow (milk diet; rhubarb; senna; santonin; unchanged bilirubin)

    (c) Green (spinach and other chlorophyllic vegetables; calomel; biliverdin)

    (d) Clay (deficiency of bile in jaundice)

    (e) "Acholic" (undigested fat; jaundice)

    (f) Dark red or chocolate brown (excess cocoa or chocolate)

    (g) Black (iron; bismuth suboxide; charcoal; blood)

    (h) Red (undigested blood; beets)

The *"separation test"* for intestinal motility is conducted by giving just before one of the meals of the day a gelatin capsule (No. 00) containing 0.2 to 0.3 of a gram of carmine or charcoal (former preferred). An inspection is then made of the subsequent stools and a note made of the time elapsing between the ingestion of the capsule and the appearance of a red (carmine) or black (charcoal) stool; normally this is about twenty-four hours.

4. *Odor,* which is normally due to indole and skatol, is greatly influenced by diet and disease. The normal odor is subject to wide variations but can usually be reported under the following designations: (a) normal; (b) slight; (c) almost odorless; (d) sour; (e) pungent; (f) putrid; (g) very offensive, etc. Odor is very marked in a meat diet, much less so from a vegetable diet and frequently hardly detectable in milk diet.

5. *Mucus* is very important and should always be reported under the following designations: (a) no excess (normal); (b) slight excess; (c) great excess; (d) almost pure mucus. It may be mixed with blood, as in dysentery; appear as firm bands suggesting tapeworms; occur as brown or black jelly-like masses, or be so

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intimately mixed with the stool as to be detected only when it is mixed with water.

6. *Concretions* are usually to be looked for while washing the stool through a sieve with water. Gallstones are faceted and, if doubtful, should be examined for cholesterin and bile pigments. Artefacts resembling stones may be composed of soap and fats following the ingestion of olive oil, etc. Intestinal concretions (enteroliths) are rare. "Intestinal sand" is usually mucus and occurs as sandlike granules. Fruit seeds (pears, apples, grapes, oranges) are sometimes mistaken for concretions until washed and closely examined.

7. *Parasites* may be readily detected by inspection, but a careful examination requires washing the stool through a sieve. Various artefacts like vegetable tissue
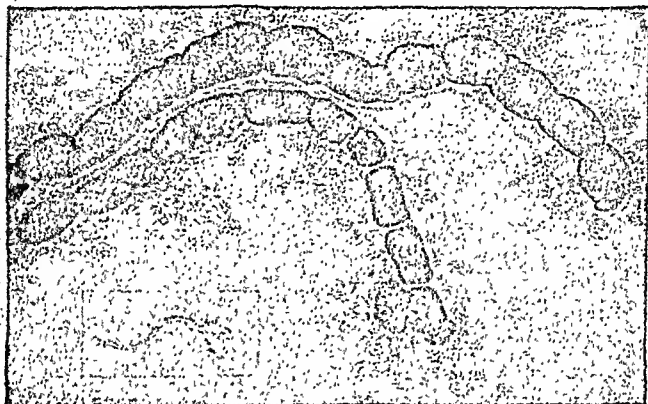
Fig. 143.—Undigested Fiber from the Center of a Banana Resembling a Parasite
(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

from poorly chewed celery or "greens," banana, fruit skins, etc., may lead to errors by hasty, incomplete examination (Fig. 143). Segments of tapeworms, round worms, pin or seat worms are usually easily detected if due care is exercised. The larvae of insects may be found in exceptional instances.

8. *Foods and curds* may be readily seen or detected after washing through a sieve. Record as slight, moderate or large numbers or amounts. The curds should be described as large or small; tough or soft, etc.

DETERMINATION OF THE REACTION

Normally the feces are slightly alkaline when freshly passed; usually they are neutral to litmus paper. An acid reaction is much less frequent and, when it does occur commonly follows a vegetable diet. Pronounced dietary changes produce at most but minor changes. Infants' stools are generally acid.

1. Examine as soon as possible after defecation.

2. Thoroughly mix the stool and test with red and blue litmus paper. If the stool is hard, mix with water.

3. Test with Congo red paper.

4. To a watery suspension add a few drops of a 1 per cent alcoholic solution of phenolphthalein (turns pink if alkaline).

DETECTION OF BLOOD

The presence of blood in the feces in appreciable amounts is often apparent on macroscopic examination. It should be noted whether the amount is sufficient to change the color of the stool (bright red, reddish, tarry), and some note should be taken also of its distribution: partially mixed, evenly mixed, only in streaks on the surface, etc.

When present in microscopic amounts, blood in the feces is termed "occult" (hidden) and detected by chemical methods.

Chemical Tests for Occult Blood.—*Principle.*—Chemical tests for occult blood depend upon the reaction of the iron of the liberated hemoglobin with the reagent in question.

Precautions.—1. In order to avoid fallacious positive reactions the patient must have been on a meat-free diet for not less than 72 hours before the collection of the specimen.

This includes abstinence from fish as well as meat and also the elimination of broths, soups, etc., made from meat stock.

2. As the blood may be unevenly distributed it is important to mix the specimen *thoroughly* before applying the test.

3. While tests may be applied to the feces directly, the reactions are more delicate and the results more reliable if applied to an extract prepared as follows:

   (a) If the stool contains much fat, extract with ether.
   (b) Mix some of the specimen with water in a mortar.
   (c) Acidify with 50% acetic acid.
   (d) Extract with ether and test the ethereal extract.

4. It is well to check a positive reaction by one method by the reaction obtained with another. The methods following appear in the order of their sensitivity.

Orthotoluidine Test.—*Reagent.*—4% orthotoluidine in glacial acetic acid.

Method.—To 1 c.c. of extract (or watery suspension of feces) add 1 c.c. of reagent and 1 c.c. of 3% hydrogen peroxide.

In the presence of blood a bluish to bluish-green color develops.

Phenolphthalein Test.—*Reagent.*

Phenolphthalein	2 grams
Potassium hydroxide	25 grams

Dissolve in 100 c.c. of distilled water, add 1 gram of powdered zinc, and heat gently until decolorized. The reagent is stable.

Method.—1. Make a thin suspension of feces in about 5 c.c. of distilled water and heat to boiling to inactivate the oxidizing enzymes.

2. Allow to cool and to 1 c.c. of reagent add 2 c.c. of the heated suspension and then add a few drops of hydrogen peroxide.

A pink to reddish color indicates a positive reaction.

Benzidine Test.—This test is most reliable when applied to the ethereal extract.

Reagents.—1. Benzidine (special for blood tests)

2. Glacial acetic acid

3. Three per cent hydrogen peroxide

Method.—1. Prepare the reagent by dissolving a knife-tip of benzidine in 2 c.c. of glacial acetic acid and adding 1 c.c. of hydrogen peroxide.

2. Make a smear of feces on a slide and pour the reagent over it (Wagner).

In the presence of blood a blue color appears, almost instantaneously if blood is present in large amounts.

DETECTION OF UROBILIN

Principles.—1. The word "urobilin" is here used as a synonym for hydrobilirubin and includes its mother substances, bilirubin and the chromogen, urobilinogen.

2. Owing to constipation and other factors, the amount is subject to variation, although the total daily output is fairly uniform.

3. Since the mother substance, bilirubin, is a product of hemoglobin, an estimation of urobilin in the feces is an approximate index of blood destruction and has had a useful application in differentiating between pernicious anemia and the secondary anemias due to hemorrhage.

4. Urobilin is absent or greatly reduced in obstructive jaundice and its return to the feces is often the first sign of relief.

Schmidt's Qualitative Test.—This test depends upon the formation of hydrobilirubin-mercury with the production of red color.

1. Rub up a small amount of feces in a mortar with a saturated watery solution of mercuric chloride.

2. Transfer to a shallow white dish and let stand for six to twenty-four hours.

3. The presence of hydrobilirubin or urobilin is indicated by a deep red color being imparted to the particles of feces containing the pigment.

If unaltered bilirubin is present, a green color is produced through its oxidation to biliverdin.

Quantitative Test of Wilbur and Addis.—This method depends upon extraction of hydrobilirubin and its quantitative estimation by spectroscopic examination.

1. Collect all the feces for twenty-four hours, keeping them in darkness.

2. Grind the whole quantity with water to a homogeneous paste.

3. Dilute to 1000 c.c. with tap water (or to 500 or 2000 c.c. if the amount of feces is unusually small or large).

4. Measure off 25 c.c. and add to this 75 c.c. acid alcohol (alcohol 64 c.c., concentrated hydrochloric acid 1 c.c., water 32 c.c.).

5. Place in a mechanical shaker for one half hour. Constant shaking by hand for a similar period will suffice.

6. Add 100 c.c. of saturated alcoholic solution of zinc acetate, and filter.

7. To 20 c.c. of the filtrate add 2 c.c. of Ehrlich's reagent (paradimethyl-

aminobenzaldehyde, 20 grams; concentrated hydrochloric acid, 150 c.c.; water 150 c.c.).

8. Keep in darkness until next day (or at least for six hours) and examine spectroscopically. In the presence of both urobilinogen and urobilin, the absorption bands indicated in Figure 141, A and B, will be seen.

9. Dilute with 60 per cent alcohol, adding a few c.c. at a time, until first one and then the other band has entirely disappeared when the slit of the
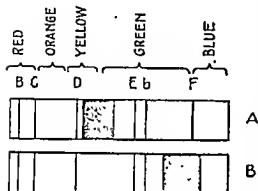
FIG. 141.—ABSORPTION SPECTRA

A, urobilinogen in acid solution with Ehrlich's reagent; B, urobilin in acid solution with zinc acetate. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

spectroscope is wide open, that still remains visible when the slit is partly closed. The end-point is fairly definite after one has established the standard upon a series of normal stools. It is perhaps best to use an unvarying width of slit and to dilute until the bands have just disappeared with this opening. One may establish uniform conditions as to the thickness of the layer of fluid, the kind and strength of the light, and the distance from the light, and then adopt a width of slit which gives an average of about 6000 dilutions in a series of normals. When using the "pocket" type of spectroscope, place the fluid in a standard serologic tube about 12 millimeters in diameter, and employ a 60-watt frosted Mazda lamp, placed about 6 inches from the spectroscope, which is mounted upon a temporary stand to insure steadiness. The eyes are protected from the light by a cardboard screen.

10. Calculate separately the number of dilutions necessary to cause disappearance of each of the absorption bands and add the two together. The calculation is based, not upon the 20 c.c. of filtrate used, *but upon the 2.5 c.c. of fecal suspension represented by the filtrate*. The dilution value for the twenty-four-hour stool (1000 c.c. of fecal suspension) is then found by multiplying this figure by 400. When the fecal suspension is made up to 500 or 2000 c.c. the multiplier would, of course, be 200 or 800. This final result indicates the number of dilutions which would be necessary if all the urobilin and urobilinogen of the twenty-four-hour stool were concentrated in the 2.5 c.c. of fecal suspension examined.

Example: Suppose that in step 9 the urobilinogen band disappeared when the 20 c.c. of filtrate had been diluted to 25 c.c., and the urobilin band when the volume reached 30 c.c., then the dilution values for the 2.5 c.c. of feces would be 10 and 12 respectively and the combined value $10 + 12 = 22$. The total dilution value of the twenty-four-hour stool would then be $22 \times 400 = 8800$.

DETECTION OF BILIRUBIN

1. Place a few drops of concentrated nitric acid in an evaporating dish or upon filter paper.

2. Apply a few drops of a suspension of feces.

3. The usual play of colors is produced (green, blue. violet, red and yellow). This test may be done on a slide and observed under a microscope.

DETECTION OF BILE ACIDS

1. Extract a small amount of feces with alcohol, and filter.

2. Evaporate the filtrate in a dish over a water bath.

3. Dissolve the residue in water made slightly alkaline with potassium hydroxide solution.

4. Add 0.3 c.c. of a 5 per cent solution of sucrose.

5. Transfer to a test tube and carefully run down the sides about 3 c.c. of concentrated sulphuric acid. Cool the tube in running water so that the temperature does not go above 70° C.

A red ring at the point of contact is a positive reaction. Upon slight agitation the contents of the tube assume a reddish color.

DETECTION OF PANCREATIC FERMENTS

Principles.—Two of the pancreatic ferments—amylase and trypsin—are normally present in feces. Lipase cannot usually be detected.

In pancreatic disease and obstruction of the pancreatic duct these ferments may be diminished or absent. Quantitative tests, therefore, may be of diagnostic value. especially in conjunction with an estimation of amylase in the urine.

Tests for both ferments should be done although that for amylase is the more useful of the two. since the action of trypsin may be simulated by erepsin and proteolytic bacteria.

Securing Specimen of Feces.—1. Upon the evening before the test, limit the patient to a light supper and give a high enema at bedtime.

2. At 7 next morning give 750 c.c. (25 ounces) of milk.

3. At 7:30 give one-half ounce Epsom salts; repeat at 8.

4. At 8:30 give a glass of water containing one-quarter teaspoonful of sodium bicarbonate.

5. Save all the feces passed up to 2 P.M. in a vessel containing 2 ounces of toluol. Keep in a cool place. If less than 400 c.c. are obtained, give an enema of 1 pint of water.

6. Dilute the whole volume of feces to 3000 c.c. with normal salt solution, mix well, and centrifugalize a portion for five minutes. Use the supernatant fluid for the following tests:

Tests for Amylase.—1. Prepare a 1 per cent solution of soluble starch as follows: To 100 c.c. of cold distilled water, add 1 gram soluble starch (Kahlbaum's recommended) and heat gently with constant stirring until clear.

2. Place 2 c.c. of this solution in each of twelve test tubes.

3. To these tubes add the supernatant fluid from the centrifugalized feces as follows:

No. 1:1.8 c.c.	No. 7:0.6 c.c.
No. 2:1.6 c.c.	No. 8:0.4 c.c.
No. 3:1.4 c.c.	No. 9:0.2 c.c.
No. 4:1.2 c.c.	No. 10:0.1 c.c.
No. 5:1.0 c.c.	No. 11:0.05 c.c.
No. 6:0.8 c.c.	No. 12:none (control)

Bring the quantity in each tube up to 4 c.c. with normal salt solution.

4. Place the tubes in an incubator or water bath at about 38° C. for one half hour.

5. Fill all tubes with tap water and add a drop of weak iodine solution to each. Gram's iodine solution will suffice.

If amylase is present, the series of tubes will vary from yellow through reddish-purple to pure blue, depending upon complete or partial digestion of the starch. The tube before the one in which the first definite trace of blue appears is taken as the measure of digestion. In normal individuals it is usually found to be either the ninth or tenth tube, corresponding to 30,000 and 60,000 units respectively.

Test for Trypsin.—The well-known Gross test may be applied as follows:

1. Prepare a 1:1000 solution of casein as follows:

Casein (C.P.)	0.1 gm.
Sodium bicarbonate	0.1 gm.
Water (Distilled)	100 c.c.

Boil for one minute, stirring constantly, and cool.

2. Place 5 c.c. of the casein solution in each of twelve test tubes and add to these tubes the same amounts of the fecal suspension, previously filtered, as were used for the amylase test.

3. Place the tubes in the incubator or a water bath at 38° C. for one hour.

4. Test for digestion of casein by adding a few drops of 3 per cent acetic acid to each tube and mixing gently. Digestion is complete in those tubes in which no white precipitate forms, and the tube before the one in which the first definite precipitate appears is taken as the measure of proteolytic activity (nearly always the fourth tube). The end-point is less definite than in the test for amylase.

DETECTION OF FATS

Fats occur as neutral fats, fatty acids and soaps, which may be differentiated as follows:

Acetic Acid Test.—By rubbing up a small portion of the feces in about 36 per cent acetic acid, applying a cover slip and heating over a flame until the preparation shows bubbles, soaps and neutral fats can be converted into free fatty acids which

show as more or less numerous highly refractile bodies and assume crystalline structure as the preparation cools. This procedure is often not successful.

TABLE I

Test	Neutral Fats	Fatty Acids	Soaps
Microscopic appearance.	Round or irregular globules; highly refractile or minute needles	Sheaves of large needles or short delicate curved needles which occur in such thick masses that the shape of the individual crystals can seldom be made out	Needles arranged in clusters or fans or in short plump crystals or scales. In amorphous form as gnarled bodies everted like the pinna of an ear. Soap crystals are comparatively coarse, as a rule (thick short needles or flakes), but may be indistinguishable from those of fatty acids
Heat	Melted	Melted	Not melted
Ether solubility........	Dissolved	Dissolved	Not dissolved
Scharlach R *	Stained	Crystals not stained. Globules stained	Not stained
Sudan III *...........	Stained	Light orange crystals not stained	
Water	0	0	Sodium and potassium soaps dissolved. Calcium and magnesium soaps not dissolved

* Scharlach R and Sudan III solutions are saturated solutions in equal parts 70 per cent alcohol and acetone

Quantitative Determination of Fats (Saxon).—The soaps are converted into free fatty acids by means of hydrochloric acid, and the material is then extracted by shaking with ether. The ether removes the neutral fat, the fatty acids present as such, the fatty acids from the soaps, and the cholesterol. The ether is removed, the crude fat purified by means of petroleum ether, and the weight of the total fat obtained. The fat is then dissolved in benzene and titrated with N/10 sodium alcoholate solution, using phenolphthalein as an indicator. The fatty acid is calculated, from the titration, as stearic acid.

1. Place about 5 grams (accurately weighed) of thoroughly mixed feces in a 100 c.c. glass-stoppered graduated cylinder, care being taken not to smear the neck of the cylinder. This procedure is best carried out by weighing a small evaporating dish of feces along with a small spatula before and after transfer.

2. Add 20 c.c. of distilled water, 1 to 2.5 c.c. of concentrated hydrochloric acid (depending upon the amount of the sample) and sufficient water to make a total bulk of 30 c.c. Add exactly 20 c.c. of ether, stopper, and shake vigorously for five

minutes. Allow to stand a few seconds, remove the stopper, add exactly 20 c.c. of 95 per cent alcohol, and again shake for five minutes.

3. Stand the cylinder aside. The ether, containing practically all of the fat, will come to the top as a colored transparent layer. Blow the ether off into a 200 c.c. Erlenmeyer flask, making use of the syphon principle as employed in the wash bottle, the submerged end of the delivery tube being bent upward. The thin layer of ether which remains is diluted with 5 c.c. of ether, the tube slightly agitated, and the ether blown off. This is done in all five times, care being taken each time to wash down the sides of the cylinder. The stopper should also be washed.

4. Twenty c.c. of ether are again added and the cylinder shaken for five minutes and set aside. When the ether has nearly stratified, blow it off and wash as before. During the second washing stratification will complete itself.

5. Using a hot water bath, distill off the ether until no trace of ether remains. This is assured by finally bringing the water bath to boil for a few minutes. To the residue add 30 c.c. of low-boiling petroleum ether. Stopper with a cork and allow to stand overnight. (Petroleum ether for this work should boil below 60° C. It should be tested for a residue on evaporation and must be redistilled if same is present.)

6. Filter the petroleum ether solution of the fat, catch the filtrate and petroleum ether washings in a tall 100 c.c. beaker which has been previously heated in an oven at 100° C., transferred to a desiccator and weighed. Evaporate off the solvent on a clean surfaced electric hot plate (being careful not to overheat near the end). Dry in an oven at 100° C., cool in a desiccator for twenty minutes and weigh. The difference in the two beaker weighings represents total fat in 5 grams of feces.

7. After weighing, dissolve the contents of the beaker in 50 c.c. of benzol, heat almost to the boiling point, add 2 drops of a 0.5 per cent solution of phenolphthalein, and titrate with an N/10 solution of sodium alcoholate.

8. The weight of fatty acids (in terms of milligrams of stearic acid) is obtained by multiplying the number of c.c. of N/10 sodium alcoholate solution by the factor 28.4.

9. The difference between the weight of the total fat and the weight of the fatty acids is the weight of the neutral fat.

NOTE.—In order to facilitate separation of the ether and water it may be desirable to put the cylinder in a centrifuge.

GENERAL MICROSCOPIC EXAMINATION

1. Prepare a thick suspension by rubbing up a portion about the size of a walnut in water (see Fig. 145). This gives a uniform mixture more representative than selecting small bits at random.

2. Place a drop on a slide and cover with a large cover glass (No. 1) for general examination.

3. Place a drop on a slide with 1 or 2 drops of 30 per cent acetic acid (No. 2) for muscle, leukocytes and pus.

4. Place a drop on a slide with 1 or 2 drops of sudan III (No. 3) for fats.

5. Place a drop on a slide with 1 or 2 drops of Lugol's solution (No. 4) for
irches.

6. Examine each microscopically with low and high power with the light well
t down as in the examination of urinary sediments.

7. In this general examination the following may be looked for (see Fig. 146):

(a) Vegetable fibers and hairs.

(b) Connective tissue consisting of colorless or yellowish threads which swell
id become gelatinous in the acetic acid preparation.

FIG. 145.—MIXING FECES WITH WATER (Benbrook)

(c) Muscle. If striations are visible, digestion is imperfect. If the nuclei are
visible, pancreatic function is absent or deficient (see Schmidt's nuclei test).

(d) Elastic tissue which generally accompanies connective tissue; outlines more
definite with branching; more distinct in the acetic acid preparation.

(e) Starch. If undigested the granules are blue on the slide treated with Lugol's
solution; reddish if partially digested.

(f) Neutral fats. Stain red with sudan III solution; also globules of fatty acids.

(g) Leukocytes and pus, which are best seen in the acetic acid preparation. A
few are normal; an excess occurs in dysentery and in other inflammatory states
masses of pure pus may be seen. In bacillary dysentery Haughwont and others have
described *macrophages* consisting of large mononuclear phagocytic cells with
large vesicular nuclei, frequently containing remnants of ingested leukocytes and
erythrocytes. They show varying degrees of necrosis and may present only circular
or oval rims with granular debris ("ghost cells"). These macrophages may be mis-
taken for amebae but can be differentiated by lack of motility and by the character
of the nuclei.

An excess of *eosinophils* may be found in the mucus in the discharges of in-
testinal allergy.

(h) Mucus, especially in mucous colitis, dysentery and other diseased states.

(i) Erythrocytes, which are best seen in the untreated slide.

(j) Epithelial cells, which show all stages of disintegration and are often unrecognizable. A marked excess of recognizable cells may occur in diseased states.

(k) Crystals, which ordinarily have but little significance: (1) slender, needle-
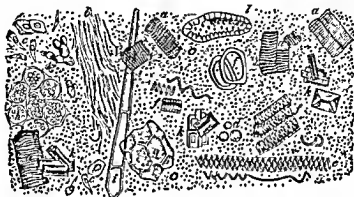
FIG. 146.—GENERAL MICROSCOPIC EXAMINATION OF FECES

(a) Muscle fibers; (b) connective tissue; (c) epithelial cells; (d) leukocytes; (e) spiral vessels of plants; (f, g, h) vegetable cells; (i) plant hairs; (k) triple phosphate crystals; (l) stone cells (after von Jaksch). (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

like crystals of fatty acids and soap; (2) triple phosphates; (3) calcium oxalate from vegetables; (4) Charcot-Leyden crystals, especially in parasitic infestments; (5) yellowish or brown needles or rhombic crystals of hematoidin after intestinal hemorrhages, etc.

(l) Cholesterin crystals and calcium bilirubinate are occasionally found and especially in cases of cholelithiasis.

Schmidt's Test Diet.—For a special microscopical examination of the digestion of muscle, starches, and fats, this test diet is recommended.

1. Bowels to be evacuated in the morning and stool discarded.

2. Breakfast of 500 c.c. (about 16 ounces) of milk and 50 grams (about 2 ounces) of toast. Also a gelatin capsule (No. 00) containing 0.2 gram of carmine as a "marker."

3. Forenoon: 0.5 liter porridge, made as follows: 40 grams oatmeal, 10 grams butter, 200 c.c. milk, 300 c.c. water, 1 egg, and salt to taste.

Midday: 125 grams hamburger steak, with 20 grams butter, fried so that the interior is quite rare; 250 grams potato, made by cooking 190 grams potato with 100 c.c. milk and 10 grams butter, the whole boiled down to 250 c.c.

Afternoon: Same as breakfast.

Evening: Same as forenoon.

4. Examine stools as soon as carmine appears as described above.

Schmidt's Nuclei Test.—This test is especially designed for the study of pancreatic function and consists of: (1) The administration of a 0.5 centimeter cube of beef or, better, of thymus, tied in a little gauze bag with the test meal. The meat must have been hardened previously in alcohol and well washed in water. (2) When the bag appears in the feces it is opened and its contents examined microscopically by pressing out small bits between a slide and cover. A drop of some nuclear stain may be applied if desired. (3) If the nuclei are for the most part undigested, pancreatic insufficiency may be assumed, since it is probable that nuclei can be digested only by the pancreatic juice. (4) Normally the nuclei are digested, provided the time of passage through the intestine is not less than eight or ten hours. Upon the other hand, if the time of passage exceeds thirty hours, nuclei may be partially digested in the complete absence of pancreatic juice.

GROSS AND MICROSCOPIC EXAMINATION FOR ANIMAL PARASITES

In the routine examination of feces for infestation with animal parasites, the following order should be adhered to:

1. Microscopic examination for trophozoites and the cysts of intestinal protozoa, particularly amebae (page 253).

2. Microscopic examination for ova or embryos of the helminthes (worms), page 268.

3. Gross examination of the entire specimen for adult parasites or segments thereof (page 287).

MICROSCOPIC EXAMINATION FOR TROPHOZOITES AND INTESTINAL PROTOZOA

Amebae.

Every routine examination of the feces for the presence of amebae should include:

(a) Examination of fresh feces for trophozoites, page 253.

(b) Examination for cysts of amebae, stained and unstained, page 255.

(c) Culture of the feces for amebae, page 261.

The laboratory diagnosis of amebiasis may also include the complement fixation test with the patient's serum. For technique, antigen, etc., see page 261.

Examination for Trophozoites (Vegetative or Motile Forms) of Amebae.
—1. Collection of suitable specimen:

Stool specimen should be collected in a warm, clean receptacle free from water, germicide or antiseptics. Patient should be cautioned not to pass urine with the feces. If the patient is passing formed stool, it is best to collect specimen after saline purge, the first or second movement following the purge being most satisfactory. During the active stage of the disease (dysentery) the fluid stool is usually satisfactory, but better material may be obtained from ulcerated areas in the sigmoid collected through the sigmoidoscope.

(1) a sealed tube of potassium **iodide** (10.0131 gms.); (2) a glass-stoppered bottle of iodine (15 gms.); a glass-stoppered volumetric flask (1000 ml.).

FIG. 149.—ENDAMOEBA COLI CYST SHOWING A LARGE VACUOLE
(After Army Medical School Collection, Washington, D. C.)

The ampule of potassium iodide is put into the volumetric flask. Add distilled water to the 1000 c.c. mark. This constitutes a one per cent solution (by weight) of potassium iodide. Add the 15 gms. of powdered iodine to this one per cent potassium iodide solution. This constitutes the stock solution, and, after it has stood for 4 days, a small portion is filtered into a stoppered dropping bottle and is ready for use. The filtered solution should be discarded after 10 days, due to volatilization of iodine.

MAYER'S HAEMALUM STAIN METHOD (*after Simmons*).—Of the various stain methods devised for the demonstration of protozoa and their cysts, the Mayer's haemalum stain is one of the most satisfactory (Fig. 150):

Nos. 12, 13, 14 and 15 are Entamoeba histolytica. Note nuclei with central karyosomes, chromatoid masses in 12, 13 and 14, and dark glycogen masses in 12 and 13. Medium size cysts are represented in 12, 13 and 14, and small size cysts in 15.
Nos. 16, 17 and 18, Endamoeba coli. Note nuclei with eccentrically situated karyosomes, and thicker nuclear membrane, and absence of the large chromatoidal masses present in the cysts of Endamoeba histolytica.
Nos. 19 and 20, Endolimax nana. Two and four nucleated cysts.
No. 21, Iodamoeba butschlii. Note large glycogen vacuole and nucleus with indistinct membrane and large, refractive, eccentric karyosome.
Nos. 22 and 23, Giardia lamblia. Note shape of cysts and presence of axostyles and parabasal bodies as refractive lines. Nuclei are four in number and minute.
Nos. 24, 25 and 26, Chilomastix mesnili. Note lemon shaped cysts containing a large nucleus with eccentrically situated karyosome and refractive shells representing cytostomal structures.
Nos. 27 and 28, Blastocystis hominis. Note nuclei situated in the outer wall which encloses a large vacuole which may contain inclusions.
No. 29, Placentozoa? Note absence of nuclei. These spores are frequently mistaken for cysts of Endamoeba histolytica but the absence of nuclei or chromatoidal bodies should serve to distinguish them. All figures are ×1675. (From Craig, Amebiasis and Amebic Dysentery, Charles C. Thomas, Springfield, Illinois.)
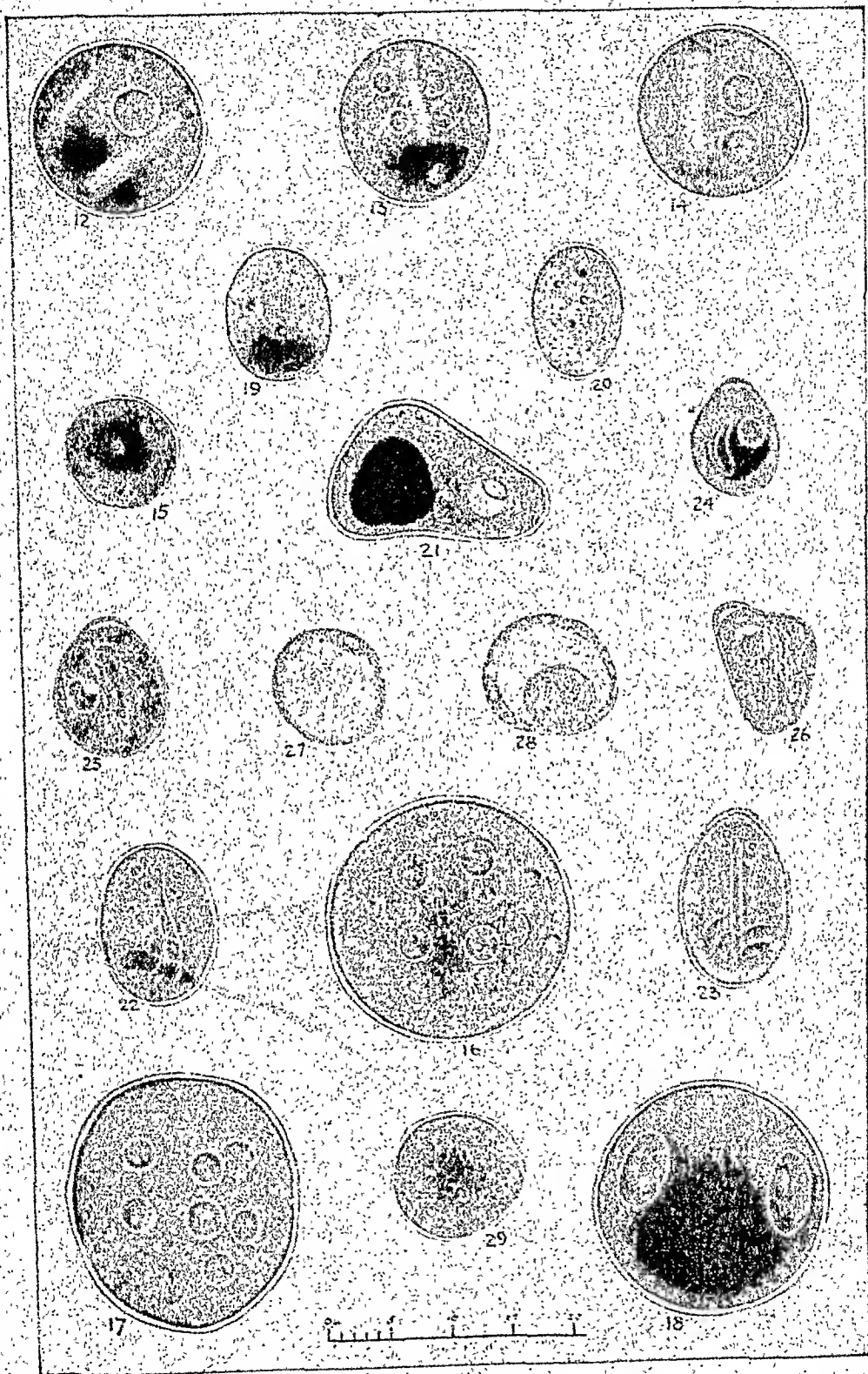
FIG. 149.—PROTOZOON CYSTS STAINED WITH IODIN STAIN·

1. Quickly smear suitable portions of the material on slides or cover glasses, and at once immerse in Schaudinn's sublimate alcohol fixing solution, previously warmed to body temperature (37.5° C.):

> Saturated bichloride of mercury......... 2 volumes
> Absolute alcohol (or 96% alcohol)..... 1 volume

Mix the alcohol and saturated solution of bichloride of mercury, and add 5% glacial acetic acid immediately before using.
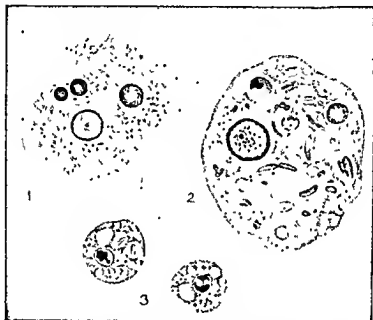
Fig. 150.—The Three Common Intestinal Amebae Stained with Iron Hematoxylin to Show Structure of Their Nuclei as Described in Table II

1, *Endamoeba histolytica* with three red blood cells; 2, *Endamoeba coli*, and 3, *Endolimax nana* (×2000). (After Dobell and O'Connor.)

If the material floats off the slide, it will be necessary to lightly coat the slide or cover glass first with egg albumin or serum before the material is spread upon it. Thick preparations should be avoided. Several smears should be prepared, since some will stain poorly.

2. Let remain in fixing solution ten to twenty minutes (10 minutes for vegetative forms, 20 minutes for cysts).

. 3. For a few minutes (5), rinse the preparation with 50 per cent alcohol to remove the sublimate.

4. Rinse for a few minutes (5) in 70 per cent alcohol with enough iodine to give a rich port wine color.

5. Dehydrate by successively passing through 50, 70, and 96 per cent alcohol. Preparations may remain in 96 per cent alcohol until it is convenient to stain them.

6. Carry the preparations successively through 70. 50 and 30 per cent alcohol, allowing them to remain five minutes in each. and then place in distilled water.

7. Immerse in the Mayer's haemalum stain from 5 to 20 minutes (5 to 10 minutes for vegetative forms, full time for cysts):

Hematoxlyn crystals	1 gm.
Sodium iodate	.2 gm.
Potassium alum	50 gm.
Distilled water	1000 c.c.

The hematoxlyn is dissolved in the distilled water, and the sodium iodate and potassium alum are then added and dissolved. The mixture is then ready for use. It should be a deep red color. When it turns brown, and when a precipitate forms. it is no longer usable.

8. Wash gently in running tap water until they appear blue in color.

9. Dehydrate by carrying the preparations successively through 30, 50, 70 90 and absolute alcohol, allowing them to remain in each five minutes.

10. Place in equal parts of absolute alcohol and zylol for five minutes.

11. Clear in xylol.

12. Mount in xylol balsam.

Caution. At no stage of the staining process must the preparations be allowed to dry.

HEIDENHAIN'S IRON HEMATOXYLIN METHOD.—This method, although more time consuming than the Mayer's haemalum stain, gives much better differentiation and is preferred by many.

1. Prepare film by smearing a small amount of material on a clean cover glass so as to form a thin, moist film.

2. Without allowing the preparation to dry, fix from 10 to 20 minutes in Schaudinn's fluid (see page 258) by floating the cover glass, film side down, on the solution which has previously been warmed (about 37° C.). The fluid may be made up in sufficient amount to fill the lower half of a Petri dish. Following the period of fixation, the preparation should be turned over so that the film side is up.

3. Drain off the fixative and cover with 50% alcohol, agitating the dish. Repeat this process several times to remove all traces of fixative. Drain and

4. Cover with 70% iodine-alcohol for 3 to 5 minutes. Drain and

5. Cover with 70% alcohol 3 to 5 minutes. Drain and

6. Cover with 85% alcohol 3 to 5 minutes. Drain and

7. Cover with 95% alcohol 3 to 5 minutes. Drain and

8. Cover with 85% alcohol 3 to 5 minutes. Drain and

9. Cover with 70% alcohol 3 to 5 minutes. Drain and

10. Cover with 50% alcohol 3 to 5 minutes. Drain and

11. Cover with 30% alcohol 3 to 5 minutes. Drain and

12. Wash in several changes of tap water for 10 to 20 minutes. Drain and

13. Cover with mordant for 15 minutes in incubator at 37° C. *Note:* Better preparations may be obtained by allowing the mordant to act over night in the incubator. (If pressed for time, *steam gently* for 15 minutes):

THE MORDANT

Ferric ammonium sulphate	4 gms.
Distilled water	100 c.c.

14. Drain off the mordant and wash in several changes of tap water 5 to 10 minutes.

15. Drain and place in 0.5% aqueous solution of hematoxylin for the same period as used in the mordant stage.

Hematoxylin (any standard hematoxylin)	0.5 gms.
Distilled water	100 c.c.

Dissolve the hematoxylin in a small amount of 95% alcohol (about 5 c.c.). Dilute to 100 c.c. with distilled water. Let ripen for at least two days before using.

16. Differentiate in fresh 2% aqueous solution of ferric ammonium sulphate (iron alum). *Note:* This is the most critical step in the whole procedure and must be done carefully by removing the preparation with forceps, dipping into the differentiating solution and quickly rinsing off in a beaker of tap water. The preparation should now be examined under the 4 mm. objective of the microscope to see whether sufficient differentiation has been accomplished. If not, the process is repeated until the nucleus stands out as a blue-black, with the cytoplasm and debris of a gray or bluish-gray shade.

17. After differentiation, wash in several changes of tap water or running water from 20 to 30 minutes.

18. Dehydrate in graded alcohols: 30%, 50%, 70%, Iodine-alcohol, 85%, 95% and absolute.

19. Clear in xylol (oil of thyme or oil of cloves may be used), 5 minutes.

20. Mount in balsam.

Heidenhain's Iron-Hematoxylin Method (Rapid method).—1. Prepare smears on cover glasses or slides; fix in methyl-alcohol for about five minutes and dry in the air.

2. Wash in water.

3. Mordant in 4% iron-alum (aqueous) for about three hours, preferably in the incubator. (In preparing this solution, select crystals of ferric ammonium sulphate that are pure violet color.)

4. Wash thoroughly in tap water.

5. Stain in ½% ripened hematoxylin for ½ hour. (Dissolve 0.5 gm. of hematoxylin in 10 c.c. of absolute alcohol and add 90 c.c. of water. Ripen for 2 or 3 months in sunlight. Process may be hastened by adding 5-10 c.c. of 0.25% $KMnO_4$).

6. Wash thoroughly in tap water.

7. Differentiate in 4% iron-alum until the nuclei are well-defined. Control under microscope, washing smears in tap water before examination.

8. Wash thoroughly in tap water, let dry in air.

9. Mount with balsam and label.

10. This method may be applied to tissues embedded in paraffin.

Detection of Cysts by Concentration Method.—When direct methods of examination for cysts are unsuccessful, it is well, before reporting specimen negative, to use one of the concentration methods as that of Craig [1]:

1. Emulsify a portion of stool about the size of a pea in 10 c.c. of water or normal salt solution (0.85%).

2. Strain the emulsion into a centrifuge tube through a suitable screen or through two layers of cheesecloth. Add sufficient salt solution to fill the tube.

3. Mix and centrifuge at moderate speed for 5 minutes.

4. With a *wide mouth pipet* secure the sediment.

5. Prepare stained and unstained preparations, and examine for cysts.

Detection of Amebae by Cultural Methods.—No examination of feces for amebae should be considered complete without culturing the specimen.

A number of media have been recommended: the Boecke-Drbohlav Locke-egg-serum medium (L. E. S. medium), Dobell and Laidlaw medium, Cleveland and Collier medium, Tenabe and Chiba's medium, Craig's medium and St. John's medium.

The Locke-egg-serum medium, one of the earliest devised, is still most generally acceptable. For constituents and technic of preparation, see Chapter XVII.

1. If the stool is solid or semi-solid, select, preferably with aseptic precautions, a portion of feces about the size of a pea; or if fluid, the specimen may be obtained with a wide-mouth pipet fitted with a rubber bulb.

2. Inoculate the L. E. S. medium, taking care to emulsify the feces thoroughly in the fluid portion thereof by rubbing the specimen against the sides of the test tube with a sterile applicator stick or inoculating needle.

3. Place in the incubator at 37.5° C. for 24 hours.

4. Remove the sediment at the bottom of the fluid portion of the medium with a *large-mouth pipet*. At least 0.1 of a c.c. should be examined.

5. Place the sediment on a warm slide, cover with a cover glass, and examine for vegetative forms.

6. A mechanical stage should be used, and every part of the preparation examined as amebae (organisms) grow quite slowly, and must be searched for thoroughly.

7. If negative results are obtained, place the tube in the incubator for a second period of 24 hour incubation, at which time a second examination should be made. Before considering a culture negative, 3 or 4 preparations should be examined.

[1] Craig, *Amebiasis and Amebic Dysentery*, Charles C. Thomas Co.

Aids to Species-Identification of Intestinal Amebae.—Five species of amebae are found in the intestine of man: (1) Endamoeba histolytica, (2) Endamoeba coli, (3) Endolimax nana, (4) Iodamoeba butschlii, (5) Dientamoeba fragilis.

The first three species are much more common than the latter two. Only the Endamoeba histolytica is pathogenic to man, producing amebic dysentery.

In the life cycle of amebae three forms may be encountered: 1. trophozoites (vegetative or motile form); 2. precyst form; 3 cyst form.

The laboratory diagnosis is established by the demonstration of the trophozoites or cysts in the feces. Care must be taken not to mistake Blastocystis hominis or the Phycomycetes for cysts of amebae. The cysts of intestinal flagellates must also be differentiated, but are usually not confused due to their characteristic appearance. (Fig. 149.)

A comparative table follows (after Craig) showing the differential characteristics of the three common amebae of man. (Table II, page 263.)

Examination of the Feces for Intestinal Flagellates.

A direct examination of the feces for flagellates may be conducted in the same manner as described for the detection of amebae. Cultural methods may be employed, as all intestinal flagellates will survive or grow on the Locke-egg or Boecke-Drbohlav media with the exception of Giardia intestinalis, which has not been successfully cultured on any medium.

Aids to the Species-Identification of the Intestinal Flagellates.—The intestinal flagellates most commonly encountered in the examination of the feces are: (1) Trichomonas hominis, (2) Chilomastix mesnili, (3) Giardia intestinalis (Lamblia intestinalis), (4) Embadomonas intestinalis, (5) Enteromonas hominis (Tricercomas intestinalis).

The life cycle of the intestinal flagellates consists of a vegetative or motile form, and a cyst stage. The vegetative form rapidly disappears after leaving the body, but the mature cyst forms are more resistant and may survive for some time. These parasites are considered by most authorities as non-pathogenic, and are found incidentally in the routine examination of the feces.

The cysts are complex in structure. They are approximately the same size as the cysts of amebae, and care must be taken not to confuse them.

A schematic representation of the important characteristics of each is shown in Figure 151.

A brief description of each is as follows:

1. Trichomonas Hominis.—This flagellate is probably the most common found in the intestine of man. It measures 9 to 15 micra. Its habitat is the large intestine, and is found in the feces, particularly during diarrhea. It may also occur in the vagina producing vaginitis. It is pear-shaped, and presents four or more flagella, three or more projecting from the anterior end, taking their origin near an oval vesicular nucleus. There is an axostyle extending from the anterior end to the posterior end projecting as a caudal process. An undulating membrane extends along the side and is edged by a flagellary process, which continues

TABLE II

Diagnostic Points in the Differentiation of Endamoeba Histolytica, Endamoeba Coli, and Endolimax Nana [2]

	Endamoeba histolytica	Endamoeba coli	Endolimax nana
	Vegetative or Trophozoite stage. Unstained.		
Size	18 to 60 micra; average, 20 to 25 micra	15 to 50 micra; average, 20 to 30 micra	6 to 12 micra, average 8 micra
Motility	Actively progressive and directional	Sluggish; rarely progressive; not directional	Sluggishly progressive
Pseudopodia	Finger-shaped, clear and glass-like	Shorter and more blunt; less glass-like in appearance	Broad and blunt; not glass-like
Inclusions	Red blood corpuscles when feces contains blood; no bacteria in fresh specimens	Numerous bacteria, crystals, and other materials; no red blood corpuscles	Numerous bacteria; no red blood corpuscles
Nucleus	Invisible	Visible	Visible
	Vegetative or Trophozoite stage. Stained.		
Nuclear membrane	Delicate; inner surface has single layer of minute chromatin dots	Thicker; inner surface lined with coarse chromatin dots	Intermediate in thickness; chromatin rarely seen on inner surface
Karyosome	Very small. Usually in center of nucleus	Twice as large, situated eccentrically	Large and may be divided into one large and one small mass, situated at one side or in center of nucleus
Intranuclear chromatin	No chromatin between karyosome and membrane	Chromatin grains between karyosome and nuclear membrane	No chromatin between karyosome and membrane
Inclusions	Red blood corpuscles; no bacteria in fresh specimens	No red blood corpuscles; many bacteria and other material	No red blood corpuscles; many bacteria
	Cystic Stage of Development. Iodine stain		
Size	6 to 20 micra; average 7 to 15 micra	10 to 20 micra; average 12 to 18 micra	5 to 10 micra
Shape	Generally spherical; may be oval and rarely irregular	Spherical; rarely oval or irregular	Spherical, oval or ellipsoidal
Nucleus	One to four; minute karyosome in center	One to eight; eccentric karyosome	One to four; large karyosome central or to one side
	Hematoxylin Stained Cysts		
Size	As in iodine-stained specimens	As in iodine-stained specimens	As in iodine-stained specimens
Nuclear structure	Delicate membrane, minute central karyosome, no chromatin between karyosome and membrane, minute grains on nuclear membrane	Thicker membrane, larger eccentrically located karyosome chromatin grains between nuclear membrane and karyosome, and large granules on nuclear membrane	Thick nuclear membrane, large central or divided karyosome
Chromatoidal bodies	Bar, oval or thick rod-like masses; present in about 50 per cent of the cysts	Filamentous or spicular with square or pointed ends; present in less than 10 percent of cysts	Small granular or bacilliform masses, not comparable with those seen in the other species
Nuclei, number of	One to four	One to eight	One to four

posteriorly. A small cystostome is present but may be obscured by structures in the anterior end. See Figure 153.

The *cyst* of this parasite has never been demonstrated. The organism is easily recognized in the feces with low power objective, and is closely related to or identical with the trichomonas found in the vagina (*Trichomonas vaginalis*) and in the mouth (*Trichomonas elongata*). In motion the organism is said to have a cog-wheel appearance. (See Fig. 152.)

2. *Chilomastix Mesnili.*—This parasite is probably the next most commonly found in the intestine of man. It measures 9 to 15 micra. It is sometimes confused
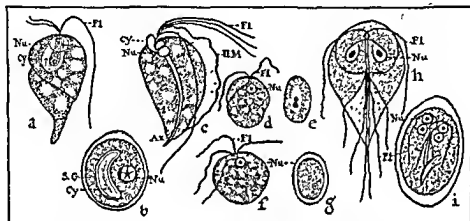
FIG. 151.—INTESTINAL FLAGELLATES (×2500)

a, Chilomastix mesnili; b, Chilomastix mesnili cyst; c, Trichomonas hominis; d, Embadomonas intestinalis; e, Embadomonas intestinalis cyst; f, Enteromonas hominis; g, Enteromonas hominis cyst; h, Giardia intestinalis; g, Giardia intestinalis cyst. (From Belding, *Manual of Human Parasitology.*)

with the Trichomonas hominis because of its similar shape. The parasite is pyriform with a tapering spine-like process in the posterior end. There is a large cystostome extending about one third of the length of the body anteriorly. Four flagella are present, three projecting anteriorly, and the fourth, which is longer, extending posteriorly. The organism inhabits the large intestine, and is occasionally found in the stool.

The cysts are oval in shape, 8½ micra in diameter. There is a large central nucleus and a laterally placed cystostome. Sometimes degenerate flagella may be seen. See Fig. 151.

3. *Giardia Intestinalis* (*Lamblia intestinalis*).—This flagellate is pear or tennis racket in shape, measuring 10x20 micra. The dorsal surface is arched. The ventral surface forms a shallow concavity which acts as a sucking disk, and enables the parasite to adhere to the intestinal wall. The structures are paired and symmetrical. Two nuclei are located near the blunt end. Four paired flagella take their origin near this point, giving the parasite a quite characteristic appearance.
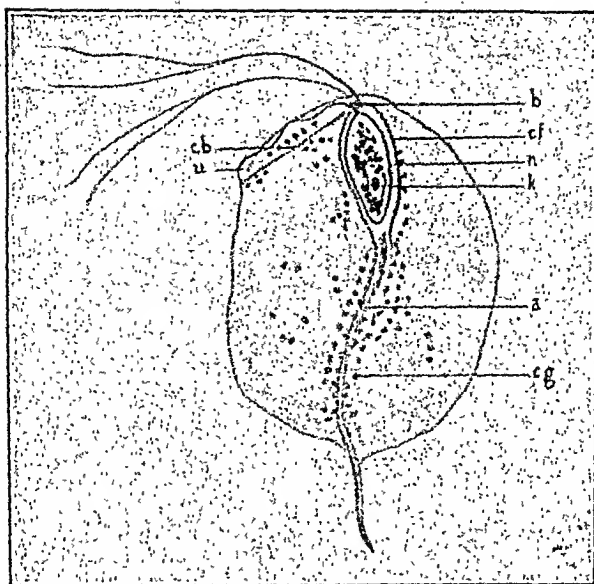
FIG. 152.—TRICHOMONAS VAGINALIS DONNÉ

Camera lucida drawing of a typical specimen ×2250, *a*, axostyle, *b*, blepharoplastic granules. *cb*, chromatic basal rod. *cf*, cytostomal fiber. *cg*, chromatic granule. *k*, karyosome. *n*, nucleus, *u*, undulating membrane. (Redrawn from Hegner.).
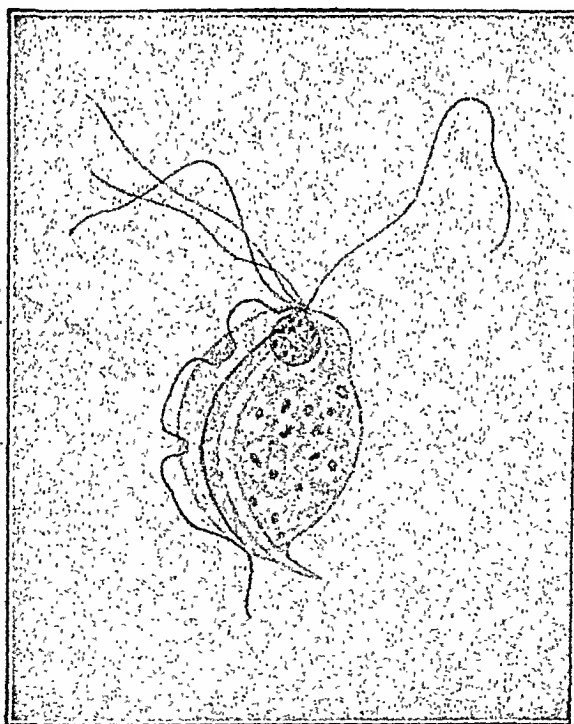
FIG. 153.—TRICHOMONAS HOMINIS.
(After Dobell and O'Connor.).

It consists of a central clear body surrounded by a narrow rim of cytoplasm which contains a number of refractile spots or nuclei. These bodies are often mistaken for cysts of protozoa. Their significance is not fully determined. They are not considered pathogenic, and are seldom reported unless they are present in unusually large numbers (Fig. 156).
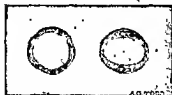
Fig 156.—Blastocystis
Hominis

A peculiar structure related to the yeasts found in feces; stained; ×1000. (After Lynch, from Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

EXAMINATION OF THE FECES FOR OVA AND LARVAE OF THE HELMINTHES (FLUKES, TAPE WORMS AND ROUND WORMS)

The laboratory diagnosis of the majority of the helminthes is accomplished by finding of the ova or embryos in the feces. The finding of even one typical ovum is sufficient to establish a diagnosis.

In the routine examination of the feces for ova, the following order should be adhered to:

1. Direct smears of the feces. If negative

2. Examination by a concentration method. If still negative

3. Examination by culture (when applicable).

Direct Smear Method for Ova.—1. Place a drop of tap water on a clean microscopic slide.

2. Take a small portion of feces from the specimen, and thoroughly emulsify in a drop of tap water.

3. Cover with a cover glass and press gently to produce a thin preparation.

4. Treat a second portion in a similar manner, substituting a drop of iodine solution for the tap water.

5. Examine under microscope using low power ocular and objective.

Note.—The iodine-stained preparation is used to avoid the missing of cysts of intestinal protozoa, such as amebae, in the routine examination for ova.

Concentration Methods for Ova.—*Shearer's Method (Benbrook Modification)*:—1. Pick up at least 1 gram of feces using a wooden tongue depressor, and place in sufficient water to liquefy it. Do not use too much water.

2. Thoroughly mix the feces with the water (Fig. 157).

3. Coarse particles may be removed if necessary by straining (Fig. 158).

4. Fill a test tube or centrifuge tube nearly half full of the fecal mixture (Fig. 159).

5. Add to the above an equal quantity of sugar solution prepared as follows:

Granulated sugar .... 1 lb.
Water .... 12 oz.

Dissolve the sugar in the water, by immersing the bottle in hot water. Add 1% phenol as a preservative.

6. Mix by slowly inverting the tube several times.
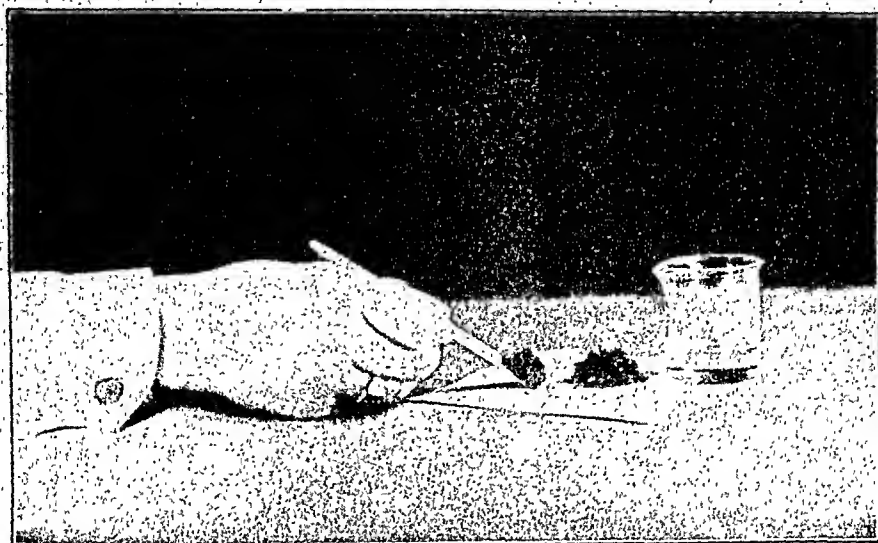
Fig. 157.—Taking a Sample of Feces (Benbrook)

Fig. 158.—Straining Feces (Benbrook)

Fig. 159.—Filling Centrifuge Tube with Fecal Suspension (Benbrook)

Fig. 160.—Lifting Surface Layer with a Headed Glass Rod (Benbrook)

7. Centrifuge the tube containing the mixture for about three minutes at moderate speed, 1500 to 2000 revolutions per minute. Centrifuging may be omitted if the tube is allowed to stand twelve to twenty-four hours.

8. Remove the tube from the centrifuge to a test tube holder without shaking.

9. Lift off the surface layer of fluid (which now contains the eggs) from the tube by means of a headed glass rod prepared as follows:

Heat one end of a 6 inch length of 5 millimeter glass rod until it is soft enough to be "headed" against a cold metal object. The head portion should be just slightly less in diameter than the inside of the tubes used (see Fig. 160). A heavy glass rod, slightly smaller than the inside of the tube, may be used in place of the headed rod. The rod should be slowly lowered into the tube and the instant full contact is made with the liquid withdraw the rod quickly, bringing with it a large drop.

10. Transfer the drop from the rod to a microscope slide by gently rotating the rod in the center of the slide. A second or third drop may be added to the first to obtain sufficient material to fill in under a micro cover slide.

11. Carefully lower a micro cover slide on the drop without pressure.

12. Examine the slide under the low power of the microscope. For best results, bright illumination should be obtained by adjusting the mirror and condenser and then modified by closing the diaphragm opening. The microscope should be vertical, not inclined, and the cover-slide area should be searched in a systematic manner. A mechanical stage is recommended.

13. When parasite eggs are seen, the high dry power should be used for identification.

Brine-Flotation Method of Kofoid and Barber.—This method is especially useful when a large number of examinations must be made, particularly for the ova of nematodes and cysts of amebae. According to McDonald, it is unsatisfactory for trematode eggs, which fail to rise to the surface.

1. A large fecal sample is thoroughly mixed with about twice its volume of saturated solution of table salt in a paraffined paste-board cup or a small beaker.

2. A lightly compressed circular disk of No. 1 or No. 0 steel wool about one-eighth to one-quarter inch thick is then placed in the cup and pushed to the bottom. This carries down all coarse particles.

3. The fluid is allowed to stand for one hour, during which time the ova rise to the surface.

4. Finally, the surface film is looped off with a wire loop about one-half inch in diameter, placed on a slide, and examined without a cover glass. The objective should be focused on the surface of the fluid.

Pepper's Method for Concentrating Hook-Worm Ova.—1. Place diluted feces on a slide and allow to remain five minutes.

2. *Gently* immerse the slide in water.

3. The ova, which have settled to the bottom, cling to the slide and are not washed away.

4. This may be repeated several times.

5. This method is not applicable to ova other than those of the hook-worm.

Method for Concentrating Operculated Ova (after Simmons).—1. Thoroughly mix a large sample of feces in 1000 c.c. distilled water.

2. Allow the mixture to settle for thirty minutes.

3. Decant and discard the sediment.

4. Allow the decanted fluid to settle for another thirty minutes.

5. Again discard the sediment and allow decanted fluid to settle ten minutes.

6. Centrifuge the decanted fluid at 1000 to 1500 revolutions per minute. The operculated ova, if present, will be found in the sediment.

Cultural Methods.—*For Schistosoma (after Simmons).*—1. Dilute a portion of specimen with water in large-mouth sterile test tube or flask.

2. Allow to stand at room temperature over night.

3. Examine a drop of fluid taken from directly beneath the pellicle on the water with pipet having a large aperture. The miracidia will escape from the egg and may be found immediately below the pellicle on the water.

For Hook-worm (Modified from Stitt).—1. Smear a generous portion of stool suspected of containing the hook-worm ova upon a filter paper small enough to be contained within a Petri dish.

2. Place two microscopic slides on the bottom of the sterile Petri dish, and lay the filter paper containing the feces on top with the buttered surface up. Sufficient tap water is added to just cover the filter paper.

3. Cover and allow to stand at room temperature for five or six days.

4. Examine fluid for the hook-worm embryos.

AIDS TO THE IDENTIFICATION OF THE MORE COMMON OVA ENCOUNTERED IN THE FECES OF MAN [a]

Operculum.—*Large,* over 50 micra, ova do not contain embryo in feces

  Ellipsoidal, rounded at both poles, large size:

    135 x 80 micra.....................Fasciolopsis buskii

    140 x 80 micra.....................Fasciola hepatica

  Abundant in sputum, less frequent in feces,

    medium size:

    95 x 55 micra.....................Paragonimus westermani

  Oval, small size:

    70 x 45 micra.....................Diphyllobothrium latum

Small, under 50 micra:

  Ovum widened at nonoperculated pole,

    giving shape of an electric light bulb'

    29 x 16 micra.....................Clonorchis sinensis

  No widening at nonoperculated pole,

    30 x 11 micra.....................Opisthorchis felineus

[a] Belding, *Manual of Human Parasitology,* Boston, Mass.

Ovoid, with slight thickening at oper-
cular rim,
    29 x 16 micra....................Heterophyes heterophyes

No Operculum.—*Wall Opaque.*

Wall thick and smooth
  Ovum elongated
    Terminal spine 140 x 50 micra. Present
      in urine, rare in feces...........Schistosoma hematobium
    Lateral spine, 150 x 50 micra.......Schistosoma mansoni
    Minute lateral knob. 85 x 60 micra....Schistosoma japonicum
  Ovum rounded, with radical striae,
  hexacanth embryo
    Ovum spherical
      35 x 35 micra...................Taenia solium
      33 x 33 micra...................Echinococcus granulosus
    Ovum ovoid, 35 x 25 micra.........Taenia saginata

Wall Transparent.

Wall thick
  Barrel-shaped, clear plugs at poles,   Trichocephalus trichiura
  52 x 23 micra......................(Trichuris trichiura)
    Globular with hexacanth larva,
    40 x 36 micra.....................Dipylidium canium
Wall thin.
  Two to four blastomeres
    70 x 38 micra...................Necator americanus
    60 x 40 micra...................Ancylostoma duodenale
  Bulge on one side, flat on other
    55 x 25 micra...................Enterobius vermicularis
  Three membranes, globular, small hexacanth
    embryo, 40 x 35 micra.............Hymenolepis nana
Wall ornamented.
  Mammillated, brown
    60 x 45 micra...................Ascaris lumbrieoides
  Ellipsoidal, three embryonic envelopes,
  shallow lozenge-shaped depression,
    90 x 50 micra.....................Macrocanthorhynchus
      hirudinaceus

AIDS TO THE IDENTIFICATION OF THE MORE COMMON HELMINTHES OF MAN

Flukes (Trematoda).

Flukes are flat, leaf-like parasitic worms. nonsegmented, attaching themselves
to the mucous membranes by suckers.

The oral sucker, located at the anterior end, leads into an esophagus which soon divides to form two caeca. The ventral sucker is found anterior to the oral sucker, its position varying with the species. Ovaries and testes are present. The vitellaria are usually prominent lateral structures.

Fasciolopsis Buskii.—Habitat the upper intestine of man. Infestation common in the Far East, particularly China. The fluke is a large oval-shaped organism, 20 to 75 mm. in length, 8 to 20 mm. in width and 2 to 3 mm. thick, opaque flesh color. The oral sucker is small. The acetabulum is large and located just posterior to the oral sucker. The testes are branched and posterior. The vitelline glands are found along the sides of the body.

Fig. 161.—Fasciola Hepatica × 5. (Wood)

The ova are large, 135 x 80 micra, brown in color, oval in shape, with a delicate operculum at one end. The contents are granular without embryo.

Fasciola Hepatica (Liver Fluke).—This fluke commonly infests sheep and other herbivorous animals. It is about 30 mm. in length, and of an opaque flesh color. It is rarely found in man (Fig. 161).

The fluke is leaf-like in shape, anteriorly terminating in a projecting cone which ends in the oral sucker. The acetabulum is located at the base of the anterior cone-like projection. Two-branched intestinal caeca are present. The testes and ovary are finely branched.

The ova are 140 x 80 micra, operculated and similar to those of *F. buskii.*

Clonorchis Sinensis (C. endemicus).—This fluke invades the ducts of the liver of man. The infestation is of importance in the Far East, particularly North China. The fluke measures 10 to 20 mm. in length, 3 to 5 mm. in width. It tapers at the anterior end. The cuticle is smooth and the fluke is almost transparent. The acetabulum is located one-fourth of the length of the body from the anterior end. Two dilated caeca are found. The branched testes are located in the posterior third of the body. Lobed ovaries in the mid-portion (Fig. 162).

The ova measure 29 by 16 micra, light brownish color. The shape is said to resemble an electric light bulb with a flattened operculum at the smaller end.

Opisthorchis Felineus.—A liver fluke of dogs and cats, occasionally infesting man. A few cases are found throughout Europe and Asia. The fluke is elongated and resembles C. sinensis. It is 7 to 12 mm. in length. It is
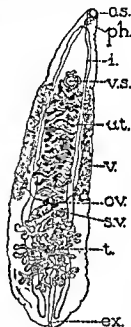
Fig. 162.—*Clonorchis sinensis.* Showing Internal Structure

o.s., oral sucker; *ph.,* pharynx; *i.,* intestine; *v.s.,* ventral sucker; *ut,* uterus, *v.,* vitellaria; *ov.,* ovary, *s.v,* vesicula seminalis; *t.,* testis; *ex,* excretory duct. *Bythinia striatula,* var. *japonica* is first intermediate host (×6). (From Ward. *Abt's Pediatrics,* W. B. Saunders Co., Philadelphia.)

differentiated from C. sinensis by the posterior location of the lobed testes, one on either side of an excretory duct.

The *ova* measure 30 by 12 micra, and are similar to those of C. sinensis.

Paragonimus Westermani (Lung Fluke).—This infestation is of importance in the Far East and in Central and South America. The organism is flat, oval shaped, reddish brown in color, 12 by 6 mm. and 5 mm. in thickness. The ventral sucker is almost centrally located. The lobed testes are located in the posterior

FIG. 163.—OVA OF SCHISTOSOMA HAEMATOBIUM WITH PUS CORPUSCLES IN URINE
(PHOTOGRAPH, ×250)

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

portion on either side of the excretory duct. The vitellaria extend laterally along the length of the body.

The *ova* are operculated, 95 x 55 micra in size. They are most frequently found in the sputum, but may be swallowed and appear in the feces. About two-fifths of patients show ova in the feces.

Schistosoma Hematobium (Blood Fluke).—This infestation is principally found in parts of Africa and the Mediterranean basin. This fluke has sex differentiation, and is long and round in shape. The male measures 10 by 15 mm. and has a ventral groove into which the slender female, 20 mm. in length lies. Both the male and female fluke are equipped with ventral and oral suckers. The male is considerably larger in diameter than the female.

The *ova* measure 140 by 55 micra in diameter, are oval and contain a fairly well developed miraeidium. The ova are deposited in the venous plexes about the urinary bladder. The ova work their way through the tissues, appearing in the urine, and in smaller numbers, in the feces. The ova are equipped with terminal spines (Fig. 163).

Schistosoma Mansoni (Blood Fluke).—The infestation is chiefly confined to parts of Africa and Northern South America. The male fluke measures 10 to 12 mm. in length, the female 12 to 16 mm. in length, and considerably less in diameter than the male.

The eggs are laid in the small veins of the intestine, reaching the feces by pene-

trating the wall. The ova measure 150 by 65 micra, and contain well-developed miracidia. They are identified by the large lateral spine (Fig. 164).

Schistosoma Japonicum.—This fluke is chiefly found in the Far East. The male measures 12 to 20 mm. in length, the female 15 to 26 mm. in length. The female is smaller in diameter than the male.

The flukes deposit the eggs in the small veins of the intestine and reach the feces by penetrating the wall. They are 85 by 60 micra in size. A well-developed miracidium is contained within. A tiny easily-overlooked spine is present (Fig. 165).

TAPEWORMS (CESTODA)

Tapeworms are flat, long, ribbon-like worms made up of a number of segments. The head is extremely small, about one mm. in diameter, and is provided with sucking disks, and in some species hooklets for attachment. The segments develop from the head and become larger as they mature.

The *proglottids* are segments which are sexually

Fig. 164.—Ovum of Schistosoma Mansoni ×460 (Morris)

mature. A tapeworm might be considered as a colony of flukes attached end to end, each segment representing a complete sexual unit, and containing both testes and ovaries. Nutrition is absorbed directly through the cuticle. The size of the worms vary from less than an inch to over 20 feet.

Man is the ultimate or definitive host for most tape worms, the eggs being passed in the feces. These are ingested by animals or fish who develop the encysted larvae in their muscles. Man acquires the disease by eating meat or fish containing the encysted embryos. In rare instances he may act as the intermediate host, by ingesting the ova (somatic taeniasis).

Taenia saginata (Beef Tapeworm).—*T. saginata* is a very common infestation of man, and is acquired by eating beef containing the encysted embryos (Cysticercus bovis).

The adult worm is from 10 to 25 feet in length, and contains from 1000 to 2000 segments. The scolex or head measures from 1 to 2 mm. in diameter. It is pear-shaped, and has four sucking disks arranged around the head laterally. No rostellum or hooklets are present.

Fig. 165.—Ovum of Schistosoma Japonicum. ×460 (Morris)

Segments.—Mature proglottids are longer than wide, about one-half inch wide, and contain a uterus with 18 to 30 lateral branches. The genital pore is lateral

and irregularly alternate (Fig. 166). These can easily be seen by placing the segment between two slides and examining by transmitted light, with the naked eye or a hand lens.

Ova.—The ova are about 35 micra in diameter, round or oval in shape. The outer covering (embryophore) is radially striated. Within is a hexacanth embryo, the hooklets of which may be recognized as six tiny paired lines (Fig. 167).
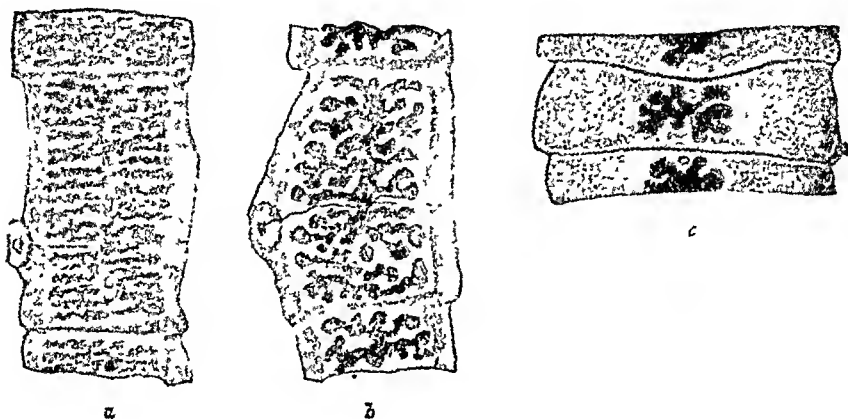
FIG. 166.—SEGMENTS OF THE THREE LARGE TAPEWORMS OF MAN, SHOWING ARRANGEMENT OF UTERUS

a. Taenia saginata; b. Taenia solium; c. Diphyllobothrium latum (×5). (From P. J. Cammidge, *The Feces of Children and Adults.*)

The laboratory diagnosis is made by the finding of the ova or segments in the feces.

Taenia Solium (Pork Tapeworm).—*T. solium* is an important tapeworm of man, but less important in this country than T. saginata. Man may suffer from both the infestation of the adult form, or, rarely, the larval stage.

The adult worm is 7 to 15 feet in length, and contains from 800 to 1000 segments. These segments are longer than they are wide. The scolex or head is globular. and about 1 mm. in diameter, and is provided with four sucking disks laterally, and a rostellum. around which are arranged 28 hooklets. The mature *proglottids* contain a uterus with 8 or 9 lateral branches. The genital pore is lateral and irregularly alternate (see Fig. 166).

The *ova* are practically identical with *T. saginata*. They are about 35 micra in diameter, and brownish in color. The outer covering is radially striated. Within, is a small hexacanth embryo (oncosphere) with three pair of hooklets.

Diagnosis is made by finding of the ova or segments in the feces. When man is the seat of *somatic taeniasis* (the embryo stage) the larvae, the cysticerci cellulosae, may be found encysted in the muscles and other organs of the body. The encysted larvae measure 5 to 10 mm. in diameter, and their laboratory diagnosis is accomplished by examining excised bits of muscle pressed between two pieces of glass, as in examination for Trichinella spiralis.

Hymenolepis Nana (Dwarf Tapeworm).—This tapeworm is a very common infestation, particularly of children.

The adult worm is small, measuring 1 to 2 inches in length, is made up of about 200 segments. The scolex is very tiny, about ⅓ of a mm. in diameter, has four sucking disks laterally placed, and has a rostellum anteriorly. Around it a row of from 24 to 30 hooklets are arranged (Fig. 168).

The mature *proglottids* are less than a mm. in width. They are identified by their
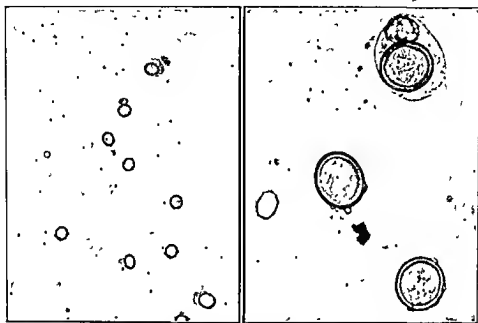
Fig. 167.—Ova of Taenia Saginata (Benbrook) ×100 and ×400

small size, three testes to each segment, and an irregular sac-like uterus which contains many ova.

The *ova* are rounded or globular in shape, about 40 micra in diameter and quite transparent. *The light must be considerably reduced in order to reveal this ovum under the microscope.* A hexacanth embryo is centrally located with three surrounding membranes. The space between the outer (vitalline membrane) and the middle (embryophore) is filled with a semi-solid material in which waxy, refractile filaments extend from each pole in a most characteristic manner.

The diagnosis is made by finding the ova in the feces. The segments may be accidentally picked up in the microscopic examination, but are too small to be detected in the gross examination of the feces (Fig. 169).

Diphyllobothrium Latum (Fish Tapeworm).—This tapeworm is of importance in certain parts of the world where fish is eaten, insufficiently cooked or raw.

The adult worm measures from 3 to 10 meters, 10 to 34 feet in length, and has
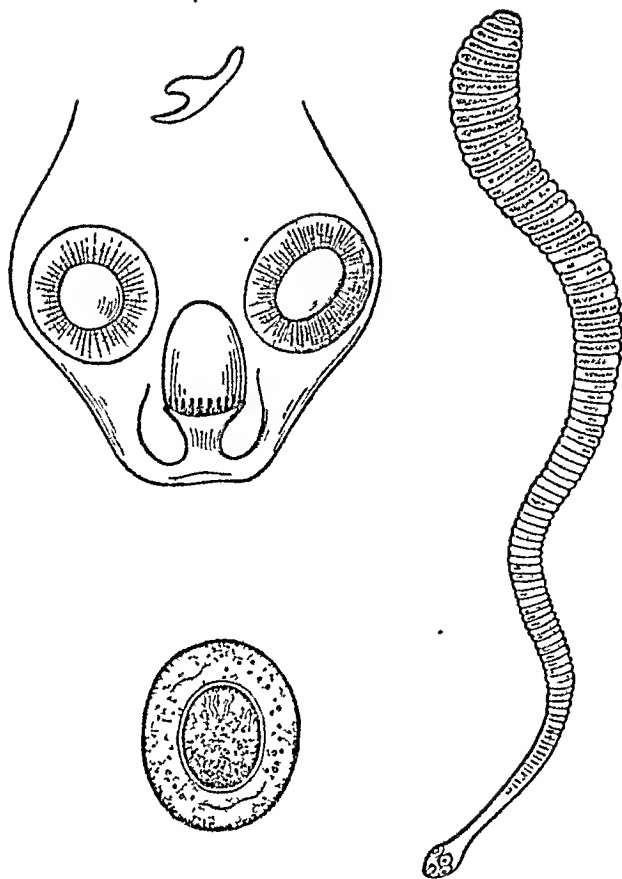
FIG. 168.—HYMENOLEPIS NANA

Parasite to the left; egg, scolex and hooklet to right. (After Braun.)
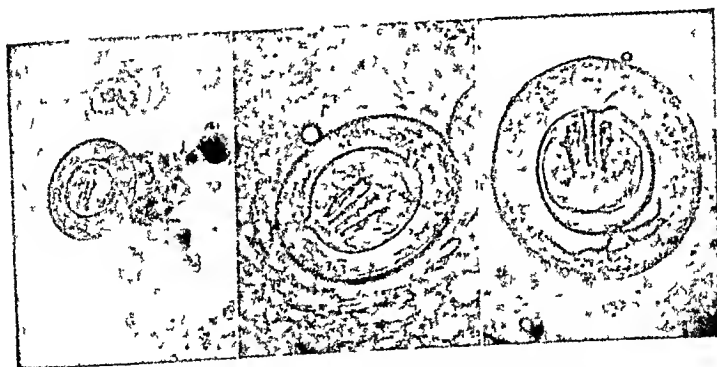
FIG. 169.—OVA OF HYMENOLEPIS NANA IN FECES

The egg to the right was compressed by pressure upon the cover glass (photograph). The figure at the left is magnified 250 diameters; the other two, 500 diameters. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

approximately 4000 segments. The scolex is almond-shaped, about 2 to 3 mm. in length, and has two lateral sucking grooves. There is no rostellum and no hooklets. The mature *segments* may be identified by the rosette-shaped centrally placed uteri

Fig. 170.—Ova of Didothriocephalus Latus (Wood)

(see Fig. 166). The segments are wider than they are long. The genital pore is in the middle of the segment.

The ova are 70 by 45 micra. They are oval in shape, somewhat brownish in color, and have a small operculum. There is no embryo within (Fig. 170).

Laboratory diagnosis is established by finding the ova or segments in the feces.

Taenia Echinococcus (Echinococcus Granulosus), Dog Tapeworm.—This infestation in man depends upon the close association between man and dogs in cattle and sheep raising countries. When man is infested he becomes the intermediate host for the parasite, the adult sexual phases taking place in the dog.

The adult worm as found in the dog is small, measuring 3 to 5 mm. in length, and consists of head and three segments. The scolex has four sucking disks, a rostellum and 38 hooklets.

The *ova* are spherical, about 33 micra in diameter, and are similar in appearance to those of Taenia solium and Taenia saginata. When the eggs are ingested
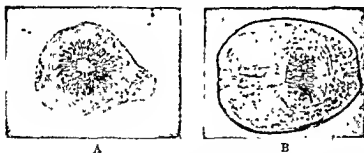
Fig. 171.—Scolices of Taenia Echinococcus from an Hepatic Cyst

A, portion of a degenerated scolex showing circle of hooklets; *B*, a well preserved scolex with crown of hooklets invaginated, a common appearance (photographs ×250). (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

by man the embryos produce echinococcic cysts in various organs, particularly the liver.

The *cyst* is formed from the body of the embryo parasite. It enlarges, and by invaginations of the germinal layer, produces daughter cysts within. These develop secondary and tertiary invaginations, finally forming scolices or heads attached

to the wall by a pedicle. The scolices have four sucking disks and a number of hooklets. Thousands are formed in each cyst (Fig. 171).

Diagnosis cannot be made from the feces. Diagnostic puncture of the cyst is attended with great danger of spreading the infection and of shock resulting from the leaking out of cyst fluid. If such puncture be made, however, the diagnosis is accomplished by finding the scolices, or in old cysts where the scolices have disintegrated, the finding of tiny chitinous hooklets.

Other diagnostic laboratory methods are complement fixation test, precipitin test and intradermal test.

Casoni Intradermal Test (after Gay).—1. Collect, exercising aseptic precautions, hydatid fluid from lung or liver of sheep showing the echinoeoceic cysts. The fluid should be checked for sterility before use in the test.

2. Inject 0.3 c.c. of the cyst fluid intracutaneously with a hypodermic syringe fitted with a fine needle.

3. A similar quantity of sterile salt solution should be injected to provide a control.

4. An urticarial wheal surrounded by a zone of erythema appears in 10 to 20 minutes, followed some hours afterward by a large area of erythema. The reaction may last 24 to 72 hours, and is usually accompanied by redness of the area and edema of the subcutaneous tissue.

Dipylidium Caninum.—This tapeworm is commonly found in dogs and cats. It occasionally infests man.

The adult worm is from 4 to 20 inches in length, and is made up of about 20 segments. The scolex is about 1/3 mm. in diameter, has 4 sucking disks, and a retractile rostellum projecting from the anterior end with 3 or 5 rows of spines or hooklets. The mature *segments* are elliptical in shape, and have a genital pore on each side.

The ova are 40 micra in diameter, slightly oval in shape, and resemble the ova of Hymenolepsis nana, except that the hexacanth embryo is much larger, and the surrounding membranes are thus closer together.

ROUND WORMS (NEMATODA)

The sexes are separate in all the nematodes. They constitute a group of important infestations of man in the United States and elsewhere.

Ascaris Lumbricoides.—The adult male is about 23 cm. in length and 0.3 cm. in diameter, the female 33 cm. in length and 0.5 cm. in diameter. They somewhat resemble the earthworm, although not segmented. They are reddish or yellowish in color when freshly passed.

The *ova* are easily recognized, measuring 60 by 45 micra, and are covered by a coarsely mammillated albuminous covering. Beneath this is a clear transparent shell enclosing an unsegmented protoplasm (Fig. 172).

The laboratory diagnosis is made by the finding of the ova in the feces, or the examination for the adults.

cavity. In the hookworm it is much larger, being as long as the entire diameter of the larvae measured at the end of the buccal cavity.

Diagnosis is made by finding the rhabditiform larvae in the fresh feces.

TRICHOCEPHALUS TRICHIURA (TRICHURUS TRICHIURA)
THE WHIPWORM

The whipworm is one of the most common intestinal infestations, having a wide geographical distribution. It is called the whipworm because of the appearance of the adult, the thick posterior two-fifths resembling a handle, the remaining anterior portion being slender and lash-like. It is chiefly found in the caecum. The male measures from 3 to 4 cm. in length, the female from 3 to 5 cm.

The ova measure 52 by 23 micra, and are very characteristic. They are brown in color, and have an inner and outer shell, with a transparent area between, and two knob-like structures at either pole (See Fig. 177).

TRICHINIASIS

Trichinella Spiralis.—This parasite is an important infestation in countries in which pork improperly cooked or raw is an article of diet. Man acquires the disease by eating pork containing the embryos.

In the intestine the adults mature. The female is 3 to 4 mm. in length, the male is smaller. In the intestine copulation takes place and the female discharges the embryos. Some enter the lymph spaces and are eventually carried to all organs of the body by the blood, *those reaching the muscles only seeming to survive.*

Fig. 178.—Trichina Spiralis Encysted in Muscle (Wood)

Here they become encysted, and can be seen with the naked eye as tiny white specks in the muscle. The cysts are lemon-shaped, measuring 400 by 250 micra, and are interposed between muscle fibers. The coiled embryo is surrounded by a capsule which it secretes. A reactionary round-celled exudate, with the formation of some fibrous tissue, may form an additional surrounding layer. See Fig. 178. After a time the cysts become calcified.

Diagnosis.—The diagnosis is made by:

1. Examination of the feces for the adult worms.

Early during the period of diarrhea the adult parasite may occasionally be demonstrated in the feces.

2. Demonstration of the larvae in the blood.

During the period of migration of the embryos from the intestine to the tissues, they may be demonstrated in the blood.

3. Demonstration of the encysted embryos in the muscle by tissue biopsy (third or fourth week of the infestation).

A small portion of the muscle may be removed from the insertion of the deltoid,

the head of the gastrocnemius, pectoralis major, or from the lower portion of the biceps, and should be divided into two parts.

The first may be examined in the fresh state by taking a small fragment (about 1 mm. in thickness) and pressing out between two small pieces of glass or micro slides so as to make the specimen translucent (preferably a Trinchina press). With a low power lens or microscope the embryo can easily be seen.

The rest of the tissue should be run through as a routine tissue biopsy and stained with hematoxylin and eosin. These muscle sections will likewise reveal the encysted embryos.

4. Intradermal and precipitin tests.

METHODS OF LABORATORY DIAGNOSIS OF INTESTINAL MYIASIS

, The larvae of several species of diptera (flies) have been reported as found in the feces. This is the result of ingestion of food containing the eggs of flies. Rarely some of the eggs may escape digestion and form larvae·which are passed in the feces. Gastro-intestinal symptoms may develop. The infestation is extremely rare. Species of bot flies. *Dermatoibia.* and flesh or meat flies, *Sarcophagga* have been reported.

The laboratory diagnosis consists of demonstrating the larval forms. the maggots, in the stool.

Caution. The diagnosis of intestinal myiasis should never be made unless the examiner is satisfied that there has been no opportunity for flies to lay their eggs or larvae in the specimen between the time of passage and that of examination. The larvae have been known to appear in a remarkably short time.

EXAMINATION OF THE FECES, GROSS AND MICROSCOPIC, FOR ADULT HELMINTHES (WORMS)

1. Place the entire stool specimen in a suitable receptacle, and add a sufficient amount of tap water to make it fluid.

2. Thoroughly mix water with the stool, and pass through a suitable screen (No. 20) to remove fluid. If the fecal matter has been properly broken up, most of it will pass through, leaving the worms or segments on the screen.

3. The material from the screen is now transferred to a clean shallow glass dish or tray (preferably with a black bottom) containing salt solution or tap water. Against this black background the parasite may·easily be seen by the use of the unaided eye.

(Note. It may be necessary to wash and screen a second time in order to free the parasites from the fecal material).

4. The parasite or segment may now be placed on a microscopic slide, covered with a cover glass, and examined with the naked eye or low power scope.

In examining tapeworm segments and flukes, it is desirable to cover with a second micro slide in place of a cover glass. By pressing the two together the specimen may be flattened. making it less opaque and thus better revealing the anatomical structure within.

Clearing the specimen with carbo-xylol (25 per cent phenol crystals and 75 per cent xylol) will also aid.

5. The specimen is best examined for internal structure by transmitted light. For details of the cuticle, or in the examination for scolices of tapeworms (to show hooklets, suckers, etc.) direct illumination or a combination of the direct illumination and transmitted light will be found advantageous.
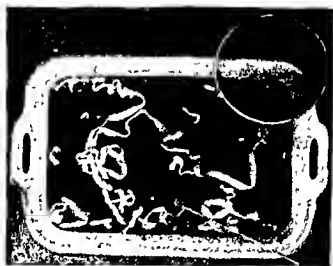
Fig. 179.—Apparatus for the Recovery of the Heads of Tapeworms
(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

6. In examining tapeworms for the head or scolex, the following procedure will be found useful:

Arrange a second tray or basin containing salt solution or tap water, adjacent to the one containing the specimen, which has been obtained by washing and screening, see paragraphs 1, 2, 3 and 4 (Fig. 179).

7. With two glass rods (about 8 inches long) as fingers feed the tapeworm into the adjacent receptacle, beginning with the larger segments and working toward the smaller.

8. Proceed in this way until the end of the worm or section thereof is reached. Examine with a lens to determine if the head is present. If not, proceed with other segments until all have been removed from the receptacle.

9. Carefully examine any remaining material in the tray for the presence of the head or scolex.

The head is very tiny, about the size of a pin head (1 mm.), the neck and adjacent segments scarcely larger than a heavy thread. They can be easily overlooked if great care is not exercised.

The finding of the head is of paramount importance to the clinician since the worm will continue to grow as long as the head remains in the intestine.

PRESERVATION OF ANIMAL PARASITES OR OVA IN THE FECES

Preservation of Worms.—*Tapeworms, Cestodes.*—1. Wash the worm free from fecal material and allow to remain an hour or two in fresh clean tap water.

2. After washing, place in ten times its volume of 3 per cent formaldehyde.

3. After twenty-four hours the formaldehyde should be changed for permanent storage.

Flukes, Trematodes.—1. Wash free from fecal material, and place in 3 per cent formaldehyde.

2. Better results may be obtained by placing the fluke between two slides to flatten out, holding the slides together with rubber bands at each end. The slides containing the specimen are then immersed in 3 per cent formaldehyde for two hours, after which the slides may be removed and the flattened specimens placed in 3 per cent formaldehyde.

Round Worms, Nematodes.—1. Round worms should be washed free from the feces, and at once placed in salt solution, since tap water will cause them to swell up and burst. They should be killed by placing in hot (70° C.) 3 per cent formaldehyde.

2. After they have been killed, by a few minutes' exposure to the hot formaldehyde, they are placed in 70 per cent alcohol for preservation.

Preservation of Feces Containing Eggs of Parasites (after Blacklock and Southwell).—1. Take a small quantity of feces (about 1 cm. in diameter) and mix with sufficient tap water to produce a semi-solid consistency.

2. Add about 200 c.c. of 10 per cent formalin (about 90° C.). Mix thoroughly by stirring. Allow to sediment for several hours.

3. Decant overlying, supernatant fluid, and add 200 c.c. of 10 per cent formalin to the sediment.

By this method worms, eggs, larvae and intestinal protozoa are fairly well preserved.

Shipment of Feces Specimens.—In the shipment of unfixed specimens of feces, the following precautions should be taken:

1. The size of the specimen should not be more than one-tenth of the capacity of the containing bottle, so as to allow for the formation and expansion of gases.

2. The cork should be firmly tied in the neck of the bottle.

3. The label should have the name of the patient, with the date passed, and how the specimen was obtained (with or without purge).

Where quantitative examination should be made, the size of the specimen in grams should be given. This may easily be obtained by weighing the specimen before and after it is placed within the receptacle.

CHAPTER XIII

METHODS FOR THE EXAMINATION OF EXUDATES AND TRANSUDATES

Principles.—1. *Transudates* are the result of noninflammatory processes and are usually due to disturbances of circulation with passive congestion and edema. The most familiar examples are pleural, pericardial and peritoneal effusions, the last named being called "ascites." The cerebrospinal fluid is also in part a transudate but is separately considered. Transudates are light yellow or serous in appearance and sometimes turn yellowish-green upon standing. At times they are milky or reddish and always of about the alkalinity of the blood.

2. *Exudates* are the result of inflammatory processes usually due to bacterial infection. As a result they vary greatly in appearance and are usually richer in cells and coagulable materials. They are likewise usually alkaline in reaction. Various types have been classified, as follows:

> (a) Serous: straw color; contains but few cells
> (b) Fibrinous: yellow; rich in fibrinogen; coagulates
> (c) Purulent: pus of various colors
> (d) Hemorrhagic: contains blood
> (e) Chylous: contains fat globules; milky
> (f) Chyloid: also milky but contains a complex of pseudoglobulin and lecithin with some fat
> (g) Putrid: usually associated with gangrene
> (h) Mixed: combinations of the above are most common

3. Aspirated fluids should be examined as soon as possible after removal. Small amounts are not sufficient for complete examinations including specific gravity, cytologic studies and guinea-pig inoculation; at least 150 to 250 c.c. should be removed whenever possible.

ROUTINE EXAMINATION

This usually embraces the following for differential diagnosis, as no one method of examination can be used alone; a combination of procedures is advisable.

1. Appearance.

2. Specific gravity, estimated according to the method employed in urine analysis.

3. Presence or absence of partial or complete coagulation and rapidity of coagulation. Exudates coagulate more rapidly and completely than transudates. The latter may not coagulate at all or show flocculi, whereas some exudates

(notably those obtained from the lungs by bronchoscopic drainage) may coagulate solid or show partial coagulation.

4. Chemical determinations, especially for quantity of protein.

5. Examination of cells (cytodiagnosis).

6. Bacteriological examination by smear, culture and animal inoculation (in suspected tuberculosis).

7. Complement-fixation tests in some instances for tuberculosis, syphilis and echinococcus disease.

8. The following table summarizes the usual differential properties of transudates and exudates.

Tests	Transudates	Exudates
Specific gravity	1.006 to 1.015 (average about 1.013). Tumor transudates, 1.018 to 1.025	Over 1.018, with average about 1.022
Coagulation	Usually absent or slight	Usually positive
Protein	Rivalta test usually negative. May be positive after concentration of fluid by absorption. Under 3 per cent in quantity	Rivalta test usually positive. Over 3 per cent in quantity
Cytology	Endothelial cells and erythrocytes. Small lymphocytes sometimes predominate. Tumor cells may be found. Eosinophils may be increased after repeated tappings	Polymorphonuclears in acute infections. Small lymphocytes in chronic infections. Eosinophils in pneumococcus infections, after repeated tappings and following artificial pneumothorax. Erythrocytes usually present
Bacteriology	Usually sterile. Staphylococcus albus from the skin may occur in cultures	Smears and cultures usually positive for pneumococci, streptococci, etc. Tubercle bacilli in smears and by guinea-pig inoculation
Complement-fixation	Positive reactions in syphilis	Positive reactions in tuberculosis and echinococcus disease

CHEMICAL EXAMINATION

1. The protein content is usually proportional to the specific gravity, that is, higher in exudates than in transudates.

2. Exudates usually show about three times as much albumin, globulin and fibrin as transudates. Albumin-globulin ratios are not sufficiently constant for differentiation.

3. Nonprotein nitrogen, urea nitrogen, creatinine, sugar, uric acid, chlorides, inorganic phosphorus and total calcium usually parallel these substances in the blood. In tumor cases the uric acid may be higher than in the blood, probably because of increased destruction of nucleoprotein. Cholesterol varies considerably.

4. Methods of quantitative estimation of these substances are the same as em-

ployed for blood analysis. For the proteins alone the following tests may be employed for differentiating between exudates and transudates.

Qualitative Acetic Acid Test (Rivalta).—1. Place 150 c.c. of distilled water in a conical flask.

2. Add 0.1 c.c. of glacial acetic acid.

3. Mix thoroughly.

4. Allow 1 or 2 drops of the puncture fluid to fall into this weak acid solution.

5. A distinct cloud will be observed in the wake of the falling drop if the fluid is an exudate. As a rule if the fluid is a transudate no turbidity will be noticed. The reaction is probably due to the large amount of a mucin-like substance, called serosomucin, especially likely to occur in exudates. Positive reactions may occur with transudates concentrated by absorption or those developing after tapping and the production of artificial pneumothorax.

Quantitative Protein Determination.—1. The protein content may be determined by the methods employed for the *quantitative estimation of protein in urine.*

2. Owing to relatively large amounts of protein likely to be present it is advisable to test the fluid diluted 1:2, 1:5 and 1:10 with saline solution. The measure of precipitate is multiplied by the dilution factor.

3. The results show grams per liter. Divide by 10 to obtain the per cent.

DIFFERENTIAL CELL COUNT

Cytodiagnosis. 1. Centrifuge *fresh* (important) specimen of the fluid. To prevent coagulation, the fluid may be collected in a little sodium citrate solution, although cytodiagnosis is better made without the use of anticoagulants.

2. Pour off supernatant fluid and make thin smears of sediment on slides. It is essential to use packed sediment in order to secure sufficient cells in smears.

3. Dry in the air.

4. Stain with Wright's or Giemsa's stain according to the method of staining blood smears.

5. Count and tabulate at least 100 of the cells. Four types may be present: lymphocytes, polymorphonuclears, eosinophils and endothelial cells. Erythrocytes in varying numbers are usually present in all fluids.

Interpretation.—1. The types of cells found depend on (a) the primary etiological factor; (b) the stage of severity of the etiological process; (c) the duration of the effusion, and (d) the secondary factors and especially the number of tappings.

2. Polymorphonuclear neutrophil leukocytes predominate in acute infectious processes, especially those due to the pyogenic organisms (Fig. 180), and may be found in early acute cases of serous tuberculous exudates.

3. *Small lymphocytes* predominate in chronic processes, especially those due to tuberculosis and syphilis (Fig. 181). They may also predominate in some chronic nontuberculous pleurisies, chronic transudates, or even tumor transudates.

4. *Eosinophils* may be present but have no clinical significance except to suggest

an allergic origin of the fluid or disease due to an animal parasite. Serous effusions caused by pneumococci may contain as high as 10 per cent eosinophils, and, according to Foord, a frequent reason for their presence is repeated aspirations

FIG. 180.—CYTODIAGNOSIS

Excess of polymorphonuclears. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

when they may be as high as 75 per cent. Differential cell counts with smears of nasal secretions for eosinophils are helpful in the diagnosis of allegeric coryza and are described in Chapter XXXI.

5. *Endothelial cells* (Fig. 182) in large numbers along with lymphocytes and erythrocytes are usually present in transudates and are largely derived from the endothelium lining the large serous cavities (pleural, peritoneal, pericardial). They commonly occur in sheets as well as singly.

6. The presence of masses of large cells, irregular in size and shape, often vacuolated, showing prominent nucleoli and sometimes mitotic figures, is highly suggestive of malignancy, but definite diagnosis is better made by sections of the imbedded sediment, in which fragments of tumor tissue, especially gland acini in adenocarcinomata, can be sometimes found. Confusion may result in smear examinations when degenerated forms of large mononucleated cells, either serosal desquamations or cells of other types ordinarily designated as macrophages, are seen.
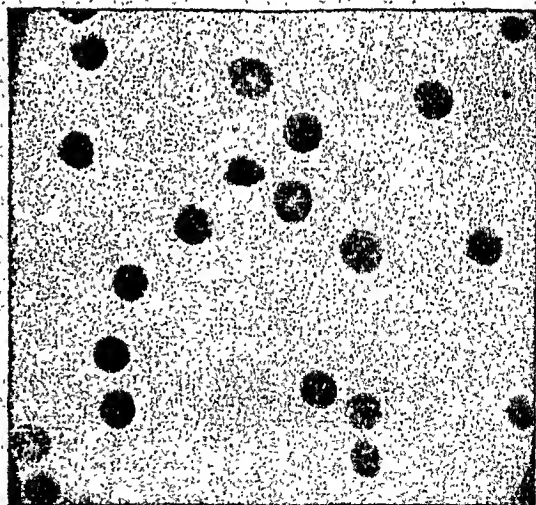
FIG. 181.—CYTODIAGNOSIS

Excess of small lymphocytes. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

Mandelbaum's Cytodiagnostic Method for Effusions.—1. Place the fluid in a large Erlenmeyer flask in the refrigerator for 12 to 18 hours.

2. Decant the supernatant fluid and transfer the sediment to a 50 c.c. centrifuge tube which has a tapered bottom.

3. Centrifuge at moderate speed for 20 minutes.

4. Decant the supernatant fluid and discard.

5. Fix the sediment with 10% formalin for 24 hours.

6. Treat the fixed sediment as a tissue running it through the alcohols and imbedding in paraffin.

7. Cut thin sections from above downward to include all of the cellular elements, which may not lie either at the top or the bottom.

8. Stain the sections with eosin and hematoxylin or iron hematoxylin. Bits of malignant tissue may be definitely demonstrated by this method in a large percentage of cases.

FIG. 182.—CYTODIAGNOSIS

Endothelial cells. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

Method for the Histological Examination of Transudates (Modified after Stanley [1]).—1. The fluid should be prepared as soon as possible after removal. In case of delay, add an equal volume of 10% formalin to fix the cells.

2. Thoroughly centrifuge a large amount (250 to 500 c.c.) in 50 c.c. centrifuge tubes.

3. Wash the sediment from each tube with 10% formalin into a small flat bottomed tube (10 c.c. homeopathic vial may be used) and centrifuge thoroughly to pack the sediment. Allow to stand for 1 or 2 days and decant the formalin.

4. Add 70% alcohol for 24 hours and centrifuge. Repeat in same manner with 95% alcohol.

5. Add absolute alcohol and place in the paraffin oven at 55° C. for about three hours, changing the alcohol once or twice.

[1] *Am. J. M. Tech.*, 1936, 2:176.

6. Remove the alcohol and add chloroform. Gently loosen the sediment so that it floats. Place in oven for 30 minutes.

7. Remove chloroform and add paraffin at 55° C. changing every half hour for 2½ hours.

8. Allow the last paraffin to harden in the tube in a refrigerator. Then heat the tube gently to loosen the paraffin slightly and remove the block (the tube may be gently broken if necessary).

9. Trim, mount on block, section and stain.

BACTERIOLOGICAL EXAMINATION

1. These methods are described in more detail in Chapter XIX.

2. Cultures should be made on blood agar or in hormone broth. Relatively large amounts of fluid, like 1 c.c., should be employed.

3. Smears of sediment should be stained by the Gram method and for tubercle bacilli.

4. Prolonged microscopic examination is usually required for the detection of tubercle bacilli.

5. In conducting the inoculation test for tubercle bacilli, at least several ounces of fluid should be centrifuged and the sediment injected into guinea-pigs. The injection of 5 c.c. amounts of fluid may yield falsely negative results.

CHAPTER XIV

METHODS FOR THE COLLECTION AND EXAMINATION OF CEREBROSPINAL FLUID

COLLECTION

1. Spinal puncture for the collection of cerebrospinal fluid may be conducted in an office or laboratory, but is better done in a hospital or the home of the patient, since it is advisable for the patient to rest in bed for at least eighteen hours immediately after the puncture as a safeguard against spinal puncture headache.

2. The *needle* should not be too large, in order to reduce pain to a minimum and to inflict the minimum of damage to the meninges. Gage No. 19 is about

FIG. 183.—SPINAL PUNCTURE IN THE SITTING POSTURE
(From Keen's *Surgery*.)

right, unless acute suppurative meningitis is suspected, in which case No. 15 may be used if a purulent and thick fluid is present. The needle should be sterilized just before use and *should be perfectly straight and sharp with a short bevel.* Crooked, rusty, dull and unnecessarily large needles are the usual causes of failure and the infliction of unnecessary pain.

3. The *sitting posture* may be used in the puncture of ambulatory adults, as shown in Figure 183, but the reclining posture with the patient lying on his

right side (Fig. 184) is recommended, especially if the spinal fluid pressure is to be taken. The latter is required in the case of children and sick adults.

4. The skin should be carefully disinfected with tincture of iodine followed by alcohol. The hands of the operator should be likewise carefully cleansed and the use of sterile rubber gloves is recommended. The operative field should be protected with sterile sheets and towels.

5. With adults the puncture can usually be made without an anesthetic. The skin may be infiltrated with sterile 1 per cent novocaine or butyn solution (Fig. 185). Struggling children and adults may require a few drops of chloroform

FIG. 184.—SPINAL PUNCTURE IN THE PRONE POSITION WITH THE BACK WELL ARCHED AND PERPENDICULAR TO THE TABLE

(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

as it is dangerous to conduct the puncture under such conditions since the needle may be broken.

6. Puncture is best conducted between the fourth and fifth or between the third and fourth lumbar vertebrae.

7. The "soft spot" between the spinous processes is located and the needle *gently and slowly* passed in the middle line. The distance varies according to the age and weight. A peculiar "give-way" sensation to the needle denotes entrance into the subarachnoid space, or during its passage the stylet may be removed from time to time to determine whether or not it has entered sufficiently as shown by flow of fluid.

8. If pure blood is obtained, the needle should be withdrawn and the needle cleansed or the puncture repeated with a fresh needle.

9. If there is no flow of fluid the needle may be gently turned or slightly withdrawn or entered a little further. "Dry taps" are usually due to the fact that the needle has not entered the subarachnoid space.

10. The pressure (if to be taken) should be taken before the escape of fluid (Fig. 186).

11. *Fluid should be collected in two sterile tubes,* one of which (No. 2) may

Fig. 185.—Producing Local Anesthesia
(From Keen's *Surgery*.)
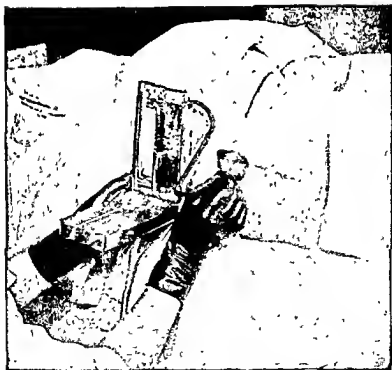
Fig. 186.—Measuring Spinal Fluid Pressure with a Mercury Manometer
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

contain a trace of powdered potassium oxalate to prevent coagulation. From 3 to 5 c.c. may be collected in No. 1 to be used for culture and the Wassermann test even if it is slightly blood-tinged. A similar amount may be collected in No. 2 to be used for the total and differential cell counts, protein and sugar determinations and the colloidal tests (gold, mastic or benzoin). This fluid should be free of blood.

12. The needle is now quickly withdrawn, the iodine removed from the skin and the puncture sealed with flexible colloidin or with an aseptic dressing. The patient should rest on the back for at least half an hour and preferably stay in bed for at least eighteen hours to reduce the chances of developing spinal puncture headache, which is believed to be due to the continued leakage of spinal fluid into the epidural space (hence the advisability of using as small a needle as possible and of reaching the fluid at the first puncture).

ORDER OF ROUTINE EXAMINATION

When Meningitis is Known or Suspected.—1. Record physical characteristics (color, transparency, coagula and sediments).

2. Then make a culture before any chances of contamination occur. Inoculate media (blood agar; glucose hormone broth, etc.) with at least 0.5 and preferably 1 c.c.

3. Shake well and do total cell count.

4. Centrifuge and prepare smears of sediment for cytological examination; stain smears by Gram method for meningococcus, streptococcus, pneumococcus, *B. influenzae* or other organisms. Stain smears for tubercle bacilli if tuberculous meningitis is suspected (also smears of coagula and inoculate guinea-pigs).

5. With supernatant fluid conduct tests for protein, sugar and chlorides (if requested).

6. Report the findings at this point.

7. Follow with a report on the cultural findings. A colloidal gold test may be conducted but is not necessary. The Wassermann reaction is not required.

When Syphilis is Known or Suspected.—1. Record physical appearance.

2. Make an *accurate* total cell count as soon as possible. A differential is not ordinarily required.

3. Conduct a qualitative test for protein (Pandy preferred).

4. Conduct Kolmer-Wassermann or Kahn tests.

5. Conduct colloidal gold (preferred), mastic, or benzoin tests.

6. Bacteriological examination is not required.

When Acute Poliomyelitis or Encephalitis Are Known or Suspected.—1. Record physical appearance.

2. Make accurate total cell count as soon as possible.

3. Centrifuge thoroughly and make cytological examination of sediment.

4. Test supernatant fluid for protein and sugar.

5. Bacteriological and Wassermann tests are not ordinarily required.

GENERAL PHYSICAL EXAMINATION

1. **Color.**—Normal spinal fluid is perfectly colorless like distilled water. Color may be recorded as follows:

  (a) Colorless
  (b) Yellow or yellowish (xanthochromia) due to altered hemoglobin, jaundice or such drugs as acriflavin
  (c) Red or reddish (erythrochromia) due to blood or hemoglobin
  (d) Greenish, grayish, etc., in meningitis

2. **Transparency.**—Normal spinal fluid is perfectly clear and transparent like distilled water. A single drop of blood in 5 c.c. may, however, render it opalescent. A classification may be made as follows:

  (a) Perfectly clear
  (b) Faintly opalescent (detected by viewing the tube against a black background)
  (c) Distinctly opalescent
  (d) Faintly turbid
  (e) Markedly turbid
  (f) Purulent

3. **Coagula and Sediments.**—Normal spinal fluid does not coagulate. In acute and chronic meningitis or passive congestion, fibrinogen may be present, which changes into fibrin with coagulation after standing. The following terminology is recommended:

  (a) No coagula
  (b) Numerous small coagula (as in paresis)
  (c) "Cobweb" or "pine-tree" coagulum (typical of tuberculous meningitis)
  (d) Heavy sunken coagulum and sediment (as in acute suppurative meningitis)

4. **Specific Gravity.**—This is not usually included in an ordinary examination. The normal varies from 1.006 to 1.008. It may be taken by the methods described for the specific gravity of urine.

5. **Reaction.**—Normally the spinal fluid is slightly alkaline with a pH of about 8.11 as compared to 7.6 to 7.8 of the blood. The reaction is not generally taken as part of a routine examination.

6. **Freezing Point.**—Normally from.—5.51° to —5.58° C. Not usually taken in routine examinations.

TOTAL CELL COUNT

Principles.—1. Whenever possible the total cell count should be made immediately after the collection of fluid while the cells are in suspension and before coagula have formed. If there is no excess of fibrin, so that coagulation does not occur, counts made some hours later or next day are fairly accurate, providing the fluid is well shaken to secure an even resuspension of cells. By collecting fluid

in a tube carrying a minute amount of potassium oxalate as described above, coagulation is prevented and counts made hours later compare quite closely with those made immediately after collection.

2. Great care in technic and accuracy are recommended because the total cell count possesses a considerable degree of diagnostic value in syphilis, acute anterior poliomyelitis and lethargic encephalitis where slight increases may occur, detectable only by accurate counts. When there is a considerable increase of total cells, as in the different types of acute suppurative and tuberculous meningitis, slight errors in counting have no particular influence upon diagnosis.

3. Spinal fluids containing visible amounts of blood are unfit for total cell counts because of the presence of leukocytes resulting in counts that are too high. Traces of blood too small for naked eye detection also increase the count very slightly but probably not to the point where the error seriously interferes with diagnosis.

FIG. 187.—FUCHS-
ROSENTHAL RULING

Procedure.—1. The Levy counting chamber with the Fuchs-Rosenthal ruling (Fig. 187) is recommended. With the cover glass on it has a depth of 0.2 millimeter with a capacity of a trifle more than 3 c.mm.

2. Draw diluting fluid to the mark 1 in the Thoma or Trenner leukocyte-counting pipets; draw spinal fluid to the mark 11.

DILUTING FLUID

Crystal violet	0.2 gm.
Glacial acetic acid	10.0 c.c.
Water (distilled)	90.0 c.c.

Filter. Should be crystal clear and free of artefacts.

3. Shake well as in leukocyte counting and discard 2 or 3 drops.

4. Fill the chamber as in leukocyte counting and wait five minutes for the cells to settle.

5. Count all of the cells (erythrocytes are hemolyzed) in the entire ruled-off area and divide by 3.5 to give the number of cells for each cubic millimeter of spinal fluid. The error incident to this calculation is practically balanced by the opposite error due to dilution.

6. If the Fuchs-Rosenthal chamber is not available, the ordinary leukocyte counting chamber may be used. In this case count the cells in the entire ruled off area (9 large squares, or 0.9 c.mm.); divide by 8 and multiply by 10. This calculation compensates for the dilution factor and gives the total cells per cubic millimeter of fluid.

7. Total cell counts are interpreted as follows:

Normal: 0 to 8 per c.mm. undiluted fluid

Border line: 9 to 12 per c.mm. undiluted fluid

Slight increase: 13 to 30 per c.mm. undiluted fluid

Moderate increase: 31 to 100 per c.mm. undiluted fluid

Great increase: hundreds to thousands per c.mm. undiluted fluid

DIFFERENTIAL CELL COUNTING AND CYTODIAGNOSIS

1. Centrifuge fresh specimen of fluid.

2. Pour off supernatant fluid and make thin smears of sediment on slides. Or tease out coagula on slides.

3. Dry in air.

4. Stain with Wright's stain previously diluted with one-third its volume of pure methyl alcohol or with a weak aqueous methylene blue stain.

5. Count and tabulate cells (lymphocytes, polymorphonuclears and endothelial cells) and determine the number of each variety per 100 cells.

6. Normally, only lymphocytes and occasional endothelial cells are found.

7. In acute suppurative meningitis due to the pneumococcus, meningococcus, streptococcus, etc., polymorphonuclear cells predominate in acute stage.

8. In tuberculous meningitis, small lymphocytes predominate (usually).

9. In acute anterior poliomyelitis, polymorphonuclears early; later small lymphocytes (usually).

10. In syphilis (paresis, tabes, etc.), small lymphocytes predominate.

11. In meningismus (serous meningitis or acute meningeal congestion), endothelial cells predominate.

QUALITATIVE DETECTION OF PROTEIN

Principles.—1. Normally the spinal fluid contains from 15 to 40 milligrams of protein per 100 c.c., with an average of about 25 milligrams or 0.025 per cent. This is largely in the form of serum globulin. Slight differences occur according to the location or level from which fluid is taken.

2. A large number of tests have been devised for the detection of an increase of protein in spinal fluid. Most of these have been for the detection of the globulins, but practically all react to some extent to serum albumin. Some were originally considered specific for syphilis of the central nervous system but none are pathognomonic for syphilis or any other disease; they merely detect an increase of protein (mostly globulins) which is always pathological unless the cerebrospinal fluid contains sufficient blood to yield positive reactions.

3. None of these tests, therefore, are applicable to cerebrospinal fluids containing macroscopic amounts of blood. Heavy bacterial contamination may likewise yield falsely positive reactions.

Pandy's Test.—1. Place about 1 c.c. of phenol reagent in a test tube.

For preparing the reagent, place 100 c.c. of pure carbolic acid (melt crystals by standing bottle in hot water) in a bottle and add water up to 1000 c.c. Shake vigorously and stand in incubator for several days. Carefully pipet off supernatant fluid or use it direct from the bottle without disturbing the layer of acid.

2. Add 1 drop of fluid to be tested.

3. If there is an increased amount of protein, a bluish white ring or cloud is immediately formed. Normal spinal fluids often show a very faint trace of globulin by this test which should not be mistaken for a positive reaction.

Ross-Jones' Test.—1. Place 1 c.c. of saturated ammonium sulphate reagent in a test tube.

The *reagent* is prepared by placing 85 grams of Merek's purified and neutral ammonium sulphate and 100 c.c. of distilled water in an Erlenmeyer flask; heat to boiling until all of the salt is dissolved. Cool slowly and filter.

2. Overlay with 1 c.c. of the fluid to be tested.

3. The appearance of a turbid ring at the point of contact—in a few seconds—indicates positive reaction. Normally a ring may appear within 5 minutes to 3 hours.

Nonne-Apelt's Test.—PHASE 1 FOR GLOBULIN.—In a small test tube place 2 c.c. of spinal fluid and 2 c.c. of the ammonium sulphate reagent (see above). Allow to stand three minutes. Compare with spinal fluid. A normal fluid gives no reaction or but a faint opalescence.

Phase 2 for Albumin.—Filter contents of tube just prepared; acidulate with acetic acid and boil. A normal fluid remains clear or but faintly opalescent.

Tryptophan Test for Tuberculous Meningitis.—1. This simple test is said to be of helpful diagnostic aid.

2. Place 2 or 3 c.c. of spinal fluid in a small test tube.

3. Add 0.5 to 0.8 c.c. of concentrated hydrochloric acid and 2 or 3 drops of 2 per cent formalin (1 c.c. of formalin and 10 c.c. distilled water).

4. Shake the tube and allow to stand for 5 minutes.

5. Carefully overlay with 2 c.c. of 0.6 per cent solution of sodium nitrate.

6. Allow to stand for 2 or 3 minutes.

7. In tuberculous meningitis a violet ring may develop at the line of contact (positive reaction). A negative reaction is shown by a brown ring, or the absence of any colored ring.

Levinson Test for Tuberculous Meningitis.—This test is based upon the principle that a characteristic ratio may be obtained between the alkaloidal precipitate formed by sulphosalicylic acid and the metallic precipitate formed by mercuric chloride in tuberculous meningitis. While positive reactions are indicative of this infection, they are not specific.

1. Into each of two small test tubes of uniform length and width place 1 c.c. of cerebrospinal fluid.

2. To one add 1 c.c. of a 3% solution of sulphosalicylic acid (C.P.) in water and to the second 1 c.c. of a 1% solution of mercuric chloride (C.P.) in water.

3. Allow to stand at room temperature for 24 hours when the sediments are measured and compared.

4. Under *normal* conditions the sediment in both tubes is very slight. In all *suppurative meningitides*, the height of the sediment in the sulphosalicylic acid tube is very heavy, often being three times the size of the sediment occurring with mercuric chloride. In *tuberculous meningitis* (rarely in other conditions) the oppo-

site occurs, the precipitation with mercuric chloride usually being three times as high as that obtained with sulphosalicylic acid.

The two precipitates are of a different character: that of the acid is heavy and compact and starts to form immediately, while that of the chloride is light, feathery, and forms slowly. Sometimes the precipitate does not come down into a compact sediment, as small flocculae may become adherent to the walls of the test tube. Under these conditions it is advisable to gently shake the tubes 2 or 3 hours before making the final readings. For diagnostic purposes it is not the amount of protein thrown down in the two precipitates, but the relative height of the sediments in millimeters in the two tubes.

If no precipitate forms, use a 2% mercuric chloride solution, and a 6% sulphosalicylic acid solution.

QUANTITATIVE ESTIMATION OF TOTAL PROTEIN

Esbach's Test.—1. Make the test in the same manner as described for urine, except that the following device is recommended for the smaller amounts of spinal fluid (Lundeberg in Simmon's *Laboratory Methods of the U. S. Army*):

2. A narrow test tube, or large glass tubing, sealed at the bottom, and measuring about 5 mm. in diameter, is strapped with adhesive tape to the side of an Esbach albuminometer tube. Spinal fluid is poured in to opposite the U mark and the reagent (trichloracetic acid, 10 c.c.; distilled water, 90 c.c.) to the R mark.

Method of Kingsbury, Clark, Williams and Post.—1. Place 1 c.c. of spinal fluid in a tube measuring ½ by 4 inches.

2. Add 3 c.c. of 3% sulphosalicylic acid and mix thoroughly.

3. Allow to stand for 5 minutes and compare the degree of turbidity with that of the permanent standard tubes. A Clark lamp aids the reading.

4. The standards may be prepared as originally described (see page 138) (*J. Lab. & Clin. M.*, 1926, 11; 981) or secured from the Fales Chemical Company, New York. They keep for some years, although they should be checked from time to time for accuracy. The normal total protein by this method is usually about 30 mg. per 100 c.c.

Sicard-Cantelouble Method.—This method employs a glass tube of 7 millimeters inside diameter, 19 centimeters long, graduated in c.c. to 4 c.c., the lower 2 c.c. being further graduated in 0.2 c.c. The method is as follows:

Place 4 c.c. spinal fluid in the tube, heat to 60° to 80° C., and add twelve drops of 33⅓% trichloracetic acid. After five minutes invert a few times. Let stand twenty-four hours, and read quantity of sediment precipitation:

1st graduation....................	**0.22** gram protein per liter
2nd graduation....................	**0.10** gram protein per liter
3rd graduation....................	**0.56** gram protein per liter
4th graduation....................	**0.71** gram protein per liter
5th graduation....................	**0.85** gram protein per liter

The normal does not exceed **0.30** gram per liter.

QUALITATIVE DETECTION OF SUGAR

Principle.—1. Normally the cerebrospinal fluid contains from 0.050 to 0.060 per cent of sugar (dextrose) which may be absent in acute suppurative meningitis and reduced in chronic meningitis (tuberculous and syphilitic).

2. Ventricular fluids are likely to contain slightly more sugar than lumbar fluids.

3. Since spinal fluid sugar varies according to the blood sugar, the fluid should be drawn after fasting overnight and compared with coincident blood sugar determinations when *quantitative* determinations are to be made.

4. Qualitative test with the Benedict reagent is essentially similar to the tests for sugar in the urine except that the reagent is diluted and slightly larger amounts of fluid are employed.

5. If the fluid being tested contains an increase of protein and an absence of sugar, the color of the reagent may be changed to a deep purplish-violet or pinkish-violet (the biuret reaction with copper).

6. Since blood sugar will give positive reactions, spinal fluids containing macroscopic amounts of blood are unfit for testing.

Procedure.—1. In a test tube place 0.5 c.c. of Benedict's *qualitative* reagent and add 4.5 c.c. of distilled water.

2. Add 1 c.c. of cerebrospinal fluid.

3. Boil for one to two minutes and allow to cool.

4. A change of color to turbid greenish-yellow is a normal reaction for the normal sugar of spinal fluid. No color change shows an absence of sugar and is pathological. An excess of protein may give a biuret reaction as mentioned above.

QUANTITATIVE DETERMINATION OF SUGAR

Principle.—Any proteins present are precipitated by tungstic acid and determination is carried out by the Folin-Wu method, using a 1:5 dilution of filtrate.

Procedure.—1. With a 1 c.c. pipet transfer 1 c.c. of cerebrospinal fluid to a clean, dry test tube.

2. Add with a pipet 3 c.c. distilled water.

3. Using a graduated 1 c.c. pipet, add 0.5 c.c. 10 per cent sodium tungstate.

4. Add 0.5 c.c. of two-thirds normal sulphuric acid.

5. Mix well and let stand five to ten minutes.

6. Filter.

7. Pipet 2 c.c. of the clear filtrate into a Folin-Wu sugar tube and proceed as with blood (see page 728).

8. Calculation, using standard 1:

$$\frac{20}{R} \times 50 = \text{milligrams dextrose per 100 c.c. fluid}$$

9. Pipet and test tubes should be absolutely clean and dry or error will be large.

10. It is advisable to make a blood sugar determination at the same time.

after preparation and then tested with a known positive spinal fluid (paretic preferred) and a known negative or normal fluid. The former should give a typical Zone 1 or paretic reaction like 5554321000 and the latter a negative reaction like 0000000000. The spinal fluids may be kept in a refrigerator for at least a month.

If a positive spinal fluid is not available the reagent may be tested with a *standard solution* of globulin by the method of Kreidler and Small as follows:

1. Prepare a 1:400 solution of edestin (H.P., Pfanstiehl) by dissolving 0.25 gram in 100 c.c. of 10% sodium chloride solution. After standing about two hours with occasional stirring filter and adjust the volume to 100 c.c. When kept in a refrigerator at 6 to 8° C., this stock solution will keep five to six months.

2. For use, dilute 1 c.c. with 24 c.c. of distilled water which gives a 1:10,000 solution of edestin in 0.4% sodium chloride. It is essential to prepare it before each titration.

3. Arrange a series of six test tubes. Place 0.1 c.c. in No. 2 and 0.2, 0.3, 0.4 and 0.5 in the remaining four tubes.

4. To each add 5 c.c. of colloidal gold reagent, mix and allow to stand for 18 hours at room temperature.

5. If the reagent is satisfactory the fourth tube carrying 0.3 c.c. of edestin solution will show a "4" reaction and the first tube (without edestin) a "0." The usual readings in the six tubes are 0, 1, 2, 4, 5 and 6.

6. If No. 4 gives less than a 4 reading and precipitation is incomplete in tubes 5 and 6, the reagent is not sufficiently sensitive and acid must be added. If No. 4 gives a 5 reading, the reagent is too sensitive and alkali must be added. In order to determine the proper amount of acid (or alkali) required for adjustment, set up a titration as follows:

(*a*) Arrange 5 test tubes and place the following amounts of N/1000 hydrochloric acid (or N/1000 sodium hydroxide): 0.1, 0.2, 0.3, 0.4 and 0.5 c.c. In rare instances it may be necessary to extend the titration series to more than five tubes, increasing the amounts of N/1000 acid (or alkali) by 0.1 c.c. in successive tubes.

(*b*) Add 0.3 c.c of the *1:1000 dilution of edestin* and 5 c.c. of colloidal gold reagent to each tube.

(*c*) Allow to stand about 18 hours at room temperature.

(*d*) A typical reading is 3, 4, 4, 5, 5. The tube containing the smallest amount of acid (or alkali) yielding a 4 reading indicates the correction to be made. In the above typical reading, the adjustment necessary for 5 c.c. of reagent would be 0.2 c.c. of N/1000 acid (or alkali) corresponding to 0.2 c.c. of N/5 acid (or N/5 alkali) for 1000 c.c. of colloidal gold reagent.

Titration of Saline Solution for the Colloidal Gold Test (Ramsay and Eilman [1]).—While the majority of reagents properly adjusted and meeting the above requirements are satisfactory in tests of spinal fluids diluted with 0.4% sodium chloride solution, this saline solution is not always satisfactory and it is advisable to determine the proper saline solution to use with each reagent as follows:

[1] *J. Lab & Clin M.*, 1932, 18:298.

1. Set up 11 test tubes and place in each 1.7 c.c. of salt solution of increasing strength, ranging from 1.0% to 2.0%. For instance: the first tube receives 1.7 c.c. of a 1.0% salt solution, the second 1.7 c.c. of a 1.1% salt solution, the third tube 1.7 c.c. of a 1.2% salt solution, and so on until the eleventh tube is reached which receives 1.7 c.c. of a 2.0% salt solution. The various salt solutions can be prepared from a 2.0% solution as follows:

Tube No. 1. 1.0 c.c. 2.0% salt solution and 1.0 c.c. distilled water
Tube No. 2. 1.1 c.c. 2.0% salt solution and 0.9 c.c. distilled water
Tube No. 3. 1.2 c.c. 2.0% salt solution and 0.8 c.c. distilled water
Tube No. 4. 1.3 c.c. 2.0% salt solution and 0.7 c.c. distilled water
Tube No. 5. 1.4 c.c. 2.0% salt solution and 0.6 c.c. distilled water
Tube No. 6. 1.5 c.c. 2.0% salt solution and 0.5 c.c. distilled water
Tube No. 7. 1.6 c.c. 2.0% salt solution and 0.4 c.c. distilled water
Tube No. 8. 1.7 c.c. 2.0% salt solution and 0.3 c.c. distilled water
Tube No. 9. 1.8 c.c. 2.0% salt solution and 0.2 c.c. distilled water
Tube No. 10. 1.9 c.c. 2.0% salt solution and 0.1 c.c. distilled water
Tube No. 11. 2.0 c.c. 2.0% salt solution and 0.0 c.c. distilled water

2. Each tube now contains 2.0 c.c. of a salt solution of the various percentages from 1.0 to 2.0%.

3. Mix well and discard 0.3 c.c. from each tube, thus leaving 1.7 c.c. in each.

4. Add to each tube 5 c.c. of the freshly prepared colloidal gold reagent.

5. The tube containing the lowest percentage of salt showing complete decolorization after one hour is used in 0.4 of this concentration for conducting the test. For instance, if decolorization of 5 c.c. colloidal gold solution occurs in the tubes containing the higher percentage of salt solution down to and including 1.2% in one hour with no decolorization below this percentage, then 0.4 of this strength, or 0.48%, is used in conducting the test. The solution should be prepared of chemically pure sodium chloride and doubly or triply distilled water.

Procedure.—1. Place eleven *chemically clean* test tubes in a rack.

2. Into the first tube place 1.8 c.c. of 0.4 *per cent sodium chloride solution* (or *the proper percentage saline as determined in the above titration*) and 1 c.c. in each of the remaining ten tubes.

3. Add 0.2 c.c. of spinal fluid to the first tube and thoroughly mix.

4. Remove 1 c.c. from the first tube and place in the second tube; mix thoroughly and remove 1 c.c. and place in the third tube; continue until the tenth tube is reached and then discard 1 c.c. from this tube. The eleventh tube is used as a control.

5. Add to each tube 5 c.c. of colloidal gold reagent.

6. Mix thoroughly and set aside for twenty-four hours, at the end of which time the readings are made.

7. Readings: Each tube is examined and the reaction recorded, using the numbers 0 to 5.

0 = unchanged as compared with the control
1 = reddish-blue
2 = lilac or purple
3 = blue
4 = almost colorless (trace of blue)
5 = colorless

8. The readings are recorded in the order in which the tubes stand (Fig. 188). For example:

5, 5, 5, 5, 4, 3, 1, 0, 0, 0 = curve in the paretic zone (Zone I) (Plate VII)

1, 1, 2, 3, 2, 1, 0, 0, 0, 0 = curve in the luetic zone (Zone II) (Plate VIII)

0, 0, 0, 1, 2, 3, 4, 5, 2, 0 = curve in the meningitic zone

0, 0, 0, 0, 0, 0, 0, 0, 0, 0 = negative

No	Color Reactions	Dilutions of Spinal Fluid									
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
5	Complete decolorization										
4	Pale blue										
3	Blue										
2	Lilac or purple										
1	Red blue										
0	No change										
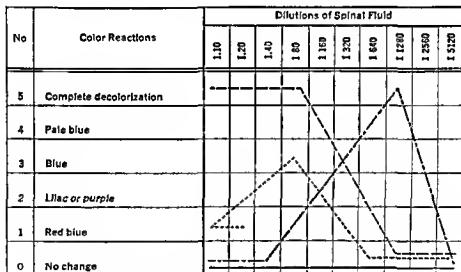
FIG. 188.—THE FOUR COMMON TYPES OF COLLOIDAL GOLD REACTIONS

————— Paretic curve or Zone I
· · · · · Luetic curve or Zone II
—·—·—· Meningitic curve
————— Negative

The chart shown in Figure 189 is recommended for reporting the results of the colloidal gold and other spinal fluid examinations.

Method of Boerner and Lukens.—This method is equally satisfactory and more economical of spinal fluid and reagent.

1. Place 1.8 c.c. of proper sodium chloride solution in the first tube and 0.5 c.c. in the remaining 10 tubes.

CEREBROSPINAL FLUID EXAMINATION

Name:
Clinical Diagnosis:

Age: Physician: Date:

Pressure (Millimeters of Mercury)	Amount Removed (C.C.)	Physical Properties	Cells per C. Mm.	Differential Cell Count				Protein Tests						Complement-fixation Reaction	
								Qualitative	Quantitative					Blood	Spinal Fluid
				Lymph.	Polys.	Endothel.		Pandy	0 2	0.1	0.05	0.025			
Before....															
After.....															

COLLOIDAL, GOLD AND MASTIC REACTIONS*

	Dilutions of Spinal Fluid										Remarks
	1 1:10	2 1:20	3 1:40	4 1:80	5 1:160	6 1:320	7 1:640	8 1:280	9 1:2560	10 1:5120	

Color Reactions

Regis-try No	Color Reactions
5	Colorless . . . .
4	Pale blue......... ... .
3	Blue . ...
2 1	Lilac or purple.. ....
1	Red-blue.... ... .
0	Red-unchanged...... .. ..

* _____ gold curve; . . . ..mastic curve.
Smears:
Dextrose:
Quantitative Kolmer Reaction:
 Serum 0.2 c.c.
 Serum 0.1 c.c.
 Serum 0.05 c.c.
 Serum 0.025 c.c.
 Serum 0.005 c.c.

Bacteriological Examination:
Culture:

Animal Inoculation:
Quantitative Chlorids:
Quantitative Kolmer Reaction:
 Spinal Fluid 0.5 c.c.
 Spinal Fluid 0.25 c.c.
 Spinal Fluid 0 125 c.c.
 Spinal Fluid 0.0625 c.c.
 Spinal Fluid 0.03125 c.c.

Examined by:

FIG. 189.—THE KOLMER CHART FOR SPINAL FLUID EXAMINATIONS.

(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

2. Add 0.2 c.c. of spinal fluid to the first tube. Mix, discard 1 c.c. and transfer 0.5 c.c. to the second tube.

3. Mix and transfer 0.5 c.c. to the third tube, and so on to the last tube, from which 0.5 c.c. is discarded after mixing.

4. Add 2.5 c.c. of reagent to all of the tubes and complete the test as described above. The readings are made in the same manner.

CUTTING'S COLLOIDAL MASTIC TEST

Principles.—1. This test depends upon the precipitation of mastic in colloidal suspension as determined by a clarification of the reagent and the production of precipitates.

2. It is highly probable that the substance in spinal fluid producing the reaction
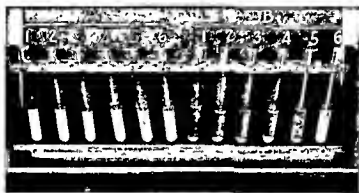
Fig. 190.—Colloidal Mastic Reactions

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

is the same as that producing the colloidal gold reaction, although its nature is unknown.

3. The reagent is much simpler and easier to prepare, although the reactions are less sensitive than the colloidal gold reaction.

4. The reaction, while less sensitive, is also less subject to technical errors.

Preparation of Reagents.—1. A stock solution of mastic is prepared by completely dissolving 10 grams of U.S.P. gum mastic in 100 c.c. of absolute alcohol. Filter.

2. For use dilute 2 c.c. with 18 c.c. of absolute alcohol, mix well, and pour rapidly into 80 c.c. of freshly distilled water.

3. Prepare a 1.25 per cent solution of C.P. sodium chloride in distilled water, and to each 99 c.c. add 1 c.c. of a 0.5 per cent solution of potassium carbonate in distilled water (alkaline-saline solution).

Procedure.—1. Arrange six small test tubes in rack.

2. Place 1.5 c.c. of alkaline-saline solution in the first tube and 1 c.c. in each of the remaining five tubes.

3. Add 0.5 c.c. of spinal fluid to the first tube, mix thoroughly and transfer 1 c.c. to the second tube.

4. Transfer 1 c.c. from the second tube to the third and so on until the fifth tube, from which 1 c.c. is discarded. The sixth tube is used as a control.

5. To each tube add 1 c.c. of mastic reagent, mix well and allow to remain at room temperature for twelve to twenty-four hours; or in the incubator for six to twelve hours.

6. A positive reaction is indicated by the formation of a heavy precipitate which settles, leaving the supernatant fluid clear (Fig. 190).

COLLOIDAL BENZOIN TEST

This test, devised by Guillain, Laroche, and Lechelle, is similar in many respects to the mastic test. It is not specific for neurosyphilis, but gives practically the same results as the more complicated colloidal gold test.

Preparation of Reagents.—1. *Benzoin Solution.*—Sumatra benzoin resin, 1 gram; absolute alcohol, 10 c.c. After forty-eight hours filter off the clear supernatant fluid. Keep in a tightly stoppered bottle. This is a stock solution from which the colloidal solution which is used in the test is freshly prepared each day as follows:

Add 0.3 c.c. of the stock benzoin solution, drop by drop with constant shaking, to 20 c.c. of doubly distilled water. Heat to 35° C. in a water bath with constant shaking.

2. *Salt Solution.*—Prepare 0.01% sodium chloride in doubly distilled water.

Procedure.—1. Set up in a rack sixteen small test tubes (75 by 10 millimeters, or 85 by 13 millimeters).

2. In the first tube place 0.25 c.c. of salt solution; in the second tube, 0.5 c.c.; in the third, 1.5 c.c., and in each of the remaining tubes 1 c.c.

3. Next add cerebrospinal fluid: 0.75 c.c. to the first tube; 0.5 c.c. to the second and third tubes. From the third tube 1 c.c. of the thoroughly mixed dilution of spinal fluid is transferred to the fourth tube, and so on, until the fifteenth tube is reached from which, after mixing, 1 c.c. is discarded. The sixteenth tube is used for control. The dilutions thus range from 3:4 in the first tube to 1:16,384 in the fifteenth tube.

4. Finally, add 1 c.c. of the benzoin suspension to each tube and mix by shaking. The tubes are allowed to stand for from eighteen to twenty-four hours.

5. The reaction will vary from no change in the mixture to complete precipitation of the benzoin, with absolute clearing of the supernatant fluid. The degree of reaction in each tube is reported: 0, no precipitation; 1, slight precipitation, with partial clearing; 2, more than half precipitated, fluid still cloudy; 3, complete precipitation, water-clear fluid. A curve may be plotted, or the figures representing the degree of reaction may be set down for each tube. Precipitation in the first six tubes indicates cerebral involvement, the first, or paretic zone; precipitation beginning with the seventh tube indicates involvement of the meninges, or spinal cord, the second, or meningeal zone. The test is not as sensitive

as in the Lange colloidal gold method, and is not as definite in its reaction in multiple sclerosis.

KOLMER-WASSERMANN REACTION

The method of conducting this reaction is described in Chapter XXIX.

BACTERIOLOGICAL EXAMINATION

1. As soon as spinal fluid is received in the laboratory, it should be cultured on blood agar or some other suitable medium before any other examinations are made, in order to avoid contamination.

2. If very cloudy, direct smears may be made on slides. If opalescent, a portion should be centrifuged and smears prepared of the sediment.

3. Smears should be stained by methylene blue and Gram's method.

4. Methods for the detection and identification of meningococci, pneumococci, streptococci, influenza and tubercle bacilli and other organisms are described in Chapter XIX.

CEREBROSPINAL FLUID IN DISEASE

The accompanying charts briefly summarize the more important changes in those diseases in which cerebrospinal fluid examinations have proved of value in diagnosis.

COMPARISON OF NORMAL CEREBROSPINAL FLUID AND BLOOD PLASMA

(Merritt and Fremont-Smith)

| | Cerebrospinal Fluid | | Plasma |
	Range	Average	Average
Specific gravity	1.006 to 1.009	1.0075	1.025
Total solids *	0.83 to 1.77	1.00	8.7
Water content *	98.23 to 99.17	99.00	91.3
Freezing point — ° C......	−0.534 to 0.603	−0.570	−0.570
Chloride †	421.0 to 454.0	410.0	360.0
Chloride (as NaCl) †	700.0 to 750.0	726.0	591.0
Bicarbonate ‡	....	21.0	23.0
Phosphorus †	1.2 to 2.1	1.5	4.0
Lactic acid †	10.0 to 20.0	15.0	15.0
Sodium †	301.0 to 313.0	324.0	316.0
Potassium †	11.0 to 15.0	13.0	19.0
Calcium †	4.5 to 5.5	5.0	10.0
Magnesium †	1.0 to 3.5	3.0	2.0
Total base ‡	....	155.0	162.0
Protein ‡	15.0 to 45.0	28.0	7000.0
Albumin	....	23.0	4430.0
Globulin	....	5.0	2270.0
Fibrinogen	....	....	300.0
Nonprotein nitrogen †	11.0 to 38.0	19.0	27.0
Urea †	8.0 to 28.0	14.0	14.0
Creatinine †	0.5 to 1.9	1.1	1.6
Amino-acid †	1.2 to 2.0	1.6	5.0
Uric acid †	0.4 to 2.8	1.7	4.7
Cholesterin †	0.06 to 0.22	0.14	160.0
Reducing substances †	50.0 to 80.0	65.0	98.0
Glucose	....	61.0	92.0
Nonglucose	....	4.0	6.0

* Grams per 100 c.c.   † Milligrams per 100 c.c.   ‡ Millimols per liter.

Summary of the Usual Cerebrospinal Fluid Changes in Disease

Disease	Pressure*	Character	Coagulation	Cytology	Qualitative Globulin	Qualitative Albumin	Quantitative Protein (Mg per 100 c.c.)	Qualitative Sugar	Quantitative Sugar (Mg per 100 c.c.)	Chlorides (Mg per 100 c.c.)	Bacteria	Wassermann Reaction	Colloidal Gold Reaction
Normal	100 to 200	Clear and colorless	Absent	0 to 8 Lymphocytes	-	-	15 to 40	+	50 to 60	720 to 750	None	Negative	Negative
Serous meningitis (meningismus)	Increased	Normal	Absent	Normal or slight increase of endothelials	-	-	Normal or slight increase	+	Normal	Normal	None	Negative	Negative
Anterior poliomyelitis	Increased	Normal or opalescent	Fibrin web (occasionally)	0 to 2000 Early Polymorphonuclears Later Lymphocytes	+	++	40 to 500 Slight increase in 30 per cent	++	40 to 120	Normal	None	Negative	Meningitic or Zone II curve ‡
Purulent meningitis	Marked increase	Slightly cloudy to thick pus, often xanthochromic	Thick coagulum	100 to 5000 Polymorphonuclears	+++	+++	Marked increase up to 5000	± to -	0 to 60	Normal or slight increase	Present	Negative	Meningitic curve
Chronic basilar meningitis	Normal or increased	Normal or opalescent, often xanthochromic	Coagulum	10 to 1000 Polymorphonuclears	+++	+++	100 to 1000	± to +	20 to 60	Normal	None or Menningococci	Negative	Meningitic curve
Tuberculous meningitis	Usually increased§	Usually clear	Fibrin web	30 to 1000 Lymphocytes (occasionally polymorphonuclears predominate)	++ to ++++	++ to ++++	100 to 1000	± to -	0 to 40	500 to 700	Tubercle bacillus	Negative	Meningitic curve‖
Epidemic encephalitis	Normal or increased	Normal, bloody or xanthochromic	Fibrin clot occasionally	10 to 200 Lymphocytes	± to +	+ to ++	30 to 200	++	40 to 120	Normal	None	Negative	Negative or Zone II curve
Brain tumor	Variable	Normal or xanthochromic	Absent	10 to 80 Lymphocytes	± to +	± to ++	20 to 200	+ to ++	40 to 100	Normal	None	Negative	Negative
Intraspinal tumor (compression syndrome)	Variable	Normal or xanthochromic	Massive coagulation	Normal to 50 Lymphocytes	± to ++++	± to ++++	60 to 1000	+	Normal or slight increase	Normal or slight increase	None	Negative	Negative
Syphilis (primary and secondary stages)	Normal	Normal	Absent	8 to 93 Lymphocytes	± to +	+ to ++	20 to 60	+	Normal	Normal	None	Variable	Negative or luetic (Zone II) Paretic (Zone II) curve rarely
Syphilis (meningovascular)	Normal or slight increase	Normal	Absent	2 to 1000 Lymphocytes 60 to 75 per cent	+ to ++	++ to +++	30 to 150	+	Normal or reduced	Normal	None	Positive	Luetic (Zone II) curve
Syphilis (tabes dorsalis)	Normal	Normal	Absent	10 to 75 Lymphocytes	± to +	+ to ++	30 to 60	+	Normal or reduced	Normal	None	Positive in 70 per cent	Luetic (Zone II) curve
Syphilis (paresis)	Normal or slight increase	Normal	Usually small coagula	30 to 200 Lymphocytes	+ to +++	++ to +++	50 to 100	+	Normal or reduced	Normal	None	Positive in 100 per cent	Paretic (Zone I) curve
Multiple sclerosis	Normal or slight increase	Normal	Absent	0 to 10 Lymphocytes	± to ++	+ to ++	20 to 60	+	Normal	Normal	None	Negative	50 per cent negative luetic (Zone II) or Paretic (Zone I) curves may occur

* Pressure is given in millimeters of water To convert to mercury, divide by 13
† Only the predominating cells are mentioned
‡ The Zone II curve often occurs during the early stages of acute poliomyelitis
§ If blocking of the foramina in the cistern occurs or the process becomes plastic the pressure may be decreased.
‖ The curve is often between the meningitic and Zone II curves.

SECTION III

BACTERIOLOGICAL, MYCOLOGICAL AND PARASITOLOGICAL METHODS

CHAPTER XV

METHODS FOR THE COLLECTION AND HANDLING OF MATERIAL FOR BACTERIOLOGICAL EXAMINATION

Principles.—*Not infrequently bacteriological examinations are rendered entirely valueless by faulty methods in the collection and handling of material.* The subject therefore is one of considerable importance in which practicing physicians especially require the advice and guidance of bacteriologists. The chief points may be summarized as follows:

1. To obtain the material as free as possible from contamination.

2. As far as is possible *to obtain exactly what it is desired to examine.* For example: In culturing pus from the ethmoid or sphenoid sinuses, a mere swabbing of the nose is not satisfactory as pus may be obtained direct from the infected areas by a rhinologist.

3. *To choose the proper culture medium* if cultures are to be made. For example: If streptococcus or pneumococcus infection is suspected in a chronic otitis media, cultures of pus on plain agar may fail and show only the more rapidly growing organisms, as staphylococci, diphtheroid bacilli, etc.

4. If mixed infection is suspected (as is usually true in chronic infections) to prepare cultures on plates (blood agar recommended) instead of on slants; or to submit the material itself or a swabbing for inoculation of plates in the laboratory.

5. To avoid soiling and contamination of containers, especially in the collection of sputum and feces.

6. To deliver material as quickly as possible to the laboratory after collection.

7. If contamination has occurred or if a defective method of collection has been used influencing the accuracy of the examination, as, for example, submitting smears that are too thin or cultures made on a wrong medium, to report the facts in order to guard against erroneous results and conclusions. For example: a culture of a sore throat on plain agar may show only staphylococci but does not exclude the possibility of diphtheria; or a culture of the eye on plain agar may show nothing but a staphylococcus and fail to grow streptococci or pneumococci if present.

8. With the exception of diphtheria (where the use of Löffler's blood medium is recommended) the most useful routine medium is blood agar plates. This medium will grow the less hardy organisms, including the hemoglobinophilic

PLATE VIII
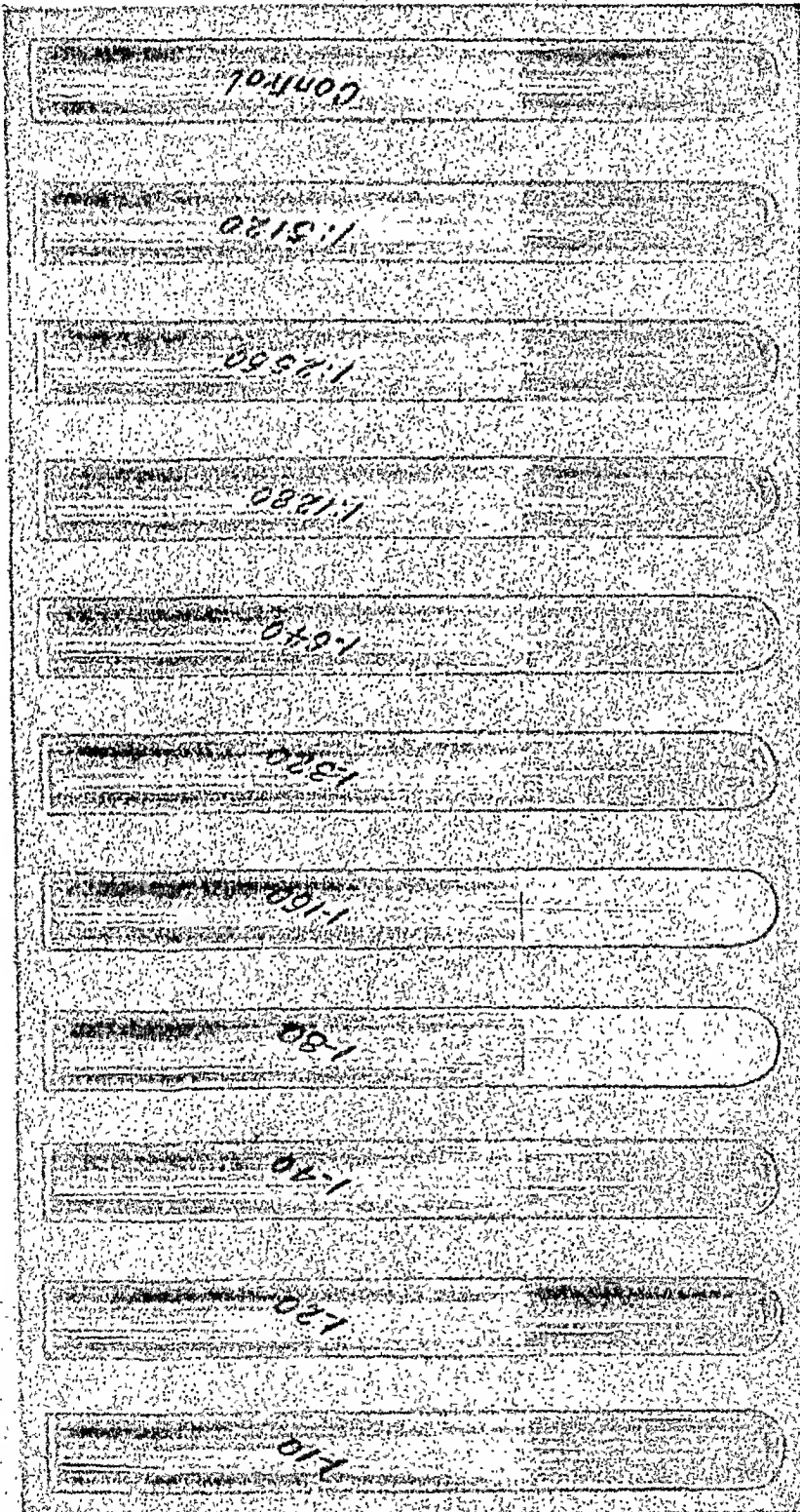
THE COLLOIDAL GOLD TEST WITH THE CEREBROSPINAL FLUID OF TABES DORSALIS, SHOWING A "LUETIC ZONE" REACTION (0 1 2-5-5 2 2 0 0 0).
(Frazier.)

5. Portions of iris removed by iridectomy should be placed at once in glucose-brain-hormone broth suitable for the cultivation of streptococci and pneumococci (pH 7.4 to 7.6).

6. *Smears are always of great value in all eye examinations* as they may show organisms failing to grow in culture media. At least two should be made. Avoid making smears too thin; smears the size of a dime are large enough.

7. In cultures of styes, plain agar may be used because these are caused by staphylococci. In all other infections use only the richer media (blood agar is recommended).

8. Do not make smears or cultures within four hours of the use of a wash or antiseptic solution.

9. Enucleated eyes should be seared or dipped in an antiseptic solution or boiling water for surface disinfection and opened with aseptic precautions for securing portions of the iris, lens, humors and retina for cultures.

COLLECTION OF MATERIAL FROM THE NOSE, SINUSES, AND NASOPHARYNX

1. In culturing the nose, sterile swabs should be used and passed without touching the atrium. They may be first passed above and then below the lower turbinates to the nasopharynx if there are no obstructions.

2. Cultures should not be made within an hour of the application of antiseptics.

3. Secretions may be blown into sterile gauze and portions picked up with sterile swabs (frequently unsatisfactory on account of contamination).

4. Material from infected sinuses should be collected by a rhinologist under direct illumination and with the aid of suction or other special methods for the purpose of securing a small amount of the material directly from the areas which are infected.

5. Cultures of the nasopharynx should be made through the mouth with curved wire swabs to avoid contamination with saliva. The West tube is useful but not necessary.

6. Plain agar should not be used except for cultures of pus from abscesses which are staphylococcic. Blood agar is recommended for routine use with Löffler's blood serum or hormone broth as second choice since rich media are required for the cultivation of streptococci, pneumococci, *Micrococcus catarrhalis*, meningococci, diphtheria bacilli and such organisms.

COLLECTION OF MATERIAL FROM TONSILS AND FAUCES

1. When inflammatory exudates are present, as in diphtheria, follicular tonsillitis and Vincent's angina, collection with sterile swabs or a sterile platinum loop is sufficient.

2. The swabbing should not be too superficial but an effort made to secure material next to the tissues. This is especially important when diphtheria is suspected, as the bacilli are apt to be deeply located while the surface of a heavy exudate shows nothing but staphylococci. For this reason the first or primary

ture may be negative for diphtheria bacilli unless a deep swabbing or a portion
membrane is secured.

. Löffler's blood serum and blood agar are recommended for the preparation
cultures.

. Smears on microslides are useful. *In Vincent's angina, smears only are re-
red* as the organisms cannot be cultivated except by very special anaerobic
thods.

. In a bacteriological examination of the tonsils in relation to focal infection,
is advisable and recommended to obtain material from the crypts whenever
ssible as these are likely to be more satisfactory than surface swabbings. As a
eral rule these collections are best made by a laryngologist. Material may be
ressed from the crypts or secured with the aid of a special sterile glass tube
ched to a suction pump. A good method and one that may be conducted in the
oratory is first to make surface swabbings in different directions, as the flora
y vary in different locations, followed by a culture of one or more crypts with
latinum loop bent at right angles.

. Excised tonsils should be delivered in sterile gauze or saline solution imme-
tely after removal. In the laboratory, they may be seared with a hot blade,
ped into boiling water or 70% alcohol for surface disinfection, washed several
es with sterile saline and laid open with a sterile knife or scissors. Cryptic
terial and bits of tissue are then secured and planted in brain-hormone broth
a similar enriched medium adapted for the cultivation of streptococci. Emul-
ns of tonsil and adenoid tissue may be prepared and cultured.

COLLECTION OF SPUTUM AND BRONCHIAL SECRETIONS

. Sputum should be collected in a sterile wide-mouthed bottle or vial with the
nimum contamination of the mouth and saliva. As a general rule, morning
tum is to be preferred, the patient being instructed to brush the teeth with a
iled toothbrush and to wash the mouth with boiled water before coughing occurs.
. In suspected *whooping cough*, sputum may be collected in this manner in
case of older children and adults. In young children, faucial secretions may be
llected on swabs. Smears are of no value. Cultures should be made on glycerol-
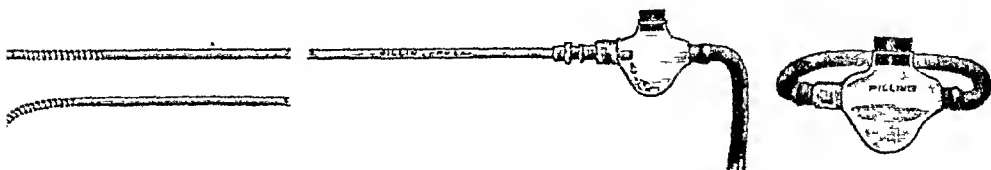
FIG. 191.—TUCKER COLLECTOR

tato-blood agar adjusted by acetic acid to pH 5.0. and plates of this medium may
held before a child during a paroxysm of coughing for making "droplet"
ltures.

3. Sputum for examination for tubercle bacilli alone by smear methods need

not be collected with these precautions. However, when cultures and guinea-pig inoculation tests for tubercle bacilli are to be conducted, they are helpful in reducing the degree of contamination.

4. Sputum to be examined for tubercle bacilli by *smear* alone need be collected in 5% phenol although this is not necessary as the specimens may be autoclaved for sterilization before examination without damage to the morphology or tinctorial properties of the bacilli. In hospitals the collection may be made in paper boxes which are later destroyed by burning, but these cannot be sterilized before examination and are not recommended. Patients should be instructed to carefully avoid contamination of the outside of containers, as the material may be dangerous to handle and bacteriologists are advised not to examine material delivered in such shape.

5. Specimens should be delivered as soon as possible and kept on ice or at a low temperature until examined. Specimens 24 hours or longer since collection are almost useless for bacteriological examination except for tubercle bacilli by the smear method; older specimens, and especially those kept at room temperature, deteriorate in value for guinea-pig inoculation and culture with increasing time.

6. Bronchial secretions aspirated with the Jackson bronchoscope are especially well adapted for bacteriological examination in cases of asthma, chronic bronchitis and bronchiectasis. The Tucker (Fig. 191) and Clerf (Fig. 192) collectors are very satisfactory. After use, the collector may be sent to the laboratory, or smears and cultures (blood agar or hormone broth preferred) prepared in the clinic.

COLLECTION OF MATERIAL FROM THE TEETH AND GINGIVAE

1. For the bacteriological examination of *extracted teeth*, the following method is recommended: (a) Wall off the operative field with sterile cotton rolls. (b) Dry off the area about the tooth with sterile gauze and apply 3 per cent tincture of iodine with care to have it penetrate well into the gingival margin about the tooth. (c) Extract, and while holding tooth in the extraction forceps, sever the apex with a cutting forceps and drop it untouched into a sterile test tube or vial. (d) Deliver as soon as possible to the laboratory, where the fragment should be cultured in a rich broth medium like glucose-brain broth; or (e) the fragment may be dropped into a sterile screw top vial containing a small amount of sterile sand and gelatin-Locke solution. In the laboratory this should be shaken for ten minutes to macerate the tissue as

Fig. 192.—Clerf Collector

much as possible and the material transferred with a sterile pipet to tubes of glucose-brain broth and streaked on blood agar plates.

2. Cultures of the *socket* may be made by: (*a*) Walling off the tooth with particular care with sterile cotton rolls to prevent contamination with saliva or the tongue; (*b*) disinfecting the gums with tincture of iodine; (*c*) extracting with forceps with sterilized beaks; (*d*) curetting with sterile curet or with a sterile cotton swab removing material for inoculation in glucose hormone broth or on blood agar plates.

3. Numerous methods have been advised for *culturing the periapical region through the root canals with the tooth in situ* but the following is recommended as a simple one for routine use: (*a*) Isolate the tooth with rubber dam. (*b*) Sterilize the coronal surface with a 3 per cent tincture of iodine. (*c*) Remove the filling with sterile instruments. (*d*) Remove filling or dressing in the root canal with sterile instruments. (*e*) Mechanically cleanse and dry the canal with sterile cotton and insert sterile paper points slightly moistened with sterile saline solution to absorb any moisture oozing into the canal; remove the points and drop them into a tube of glucose hormone broth. (*f*) If no moisture oozes in, pass a sterile, fine broach or pick through the canal and drop it into a tube of the medium.

A more elaborate method recommended by Rickert is as follows: The canals are first opened large enough to be readily accessible. The canal walls should be cleansed with alcohol or hydrogen peroxide. The treatment is introduced on an aseptic cotton point of a length not to exceed two-thirds the length of the canal; above this toward the occlusal orifice place a short section of the dry thickened end of a sterile cotton point; then above this place cotton moistened with sandarac varnish. The cavity is next sealed with either cement or temporary filling. In taking the culture, the tooth and adjacent teeth are isolated, dried and treated with tincture of iodine. The temporary stopping is removed and the cavity moistened with iodine; the sandaric varnish stopping is then removed and this portion cleansed with alcohol. The last dry pledget is then removed with a barbed broach and the dressing to be cultured is carefully withdrawn; it is seized just above the point of contact of the broach with sterile cotton pliers. The broach, cut off to the pliers and the remaining part of the point which is the apical end, is then introduced into the culture medium.

4. Smears are generally employed for the bacteriological examination of the *gingivae or gums*. Material should be especially collected from the sulci or pockets alongside of the teeth in infection of the periodontium. A stiff platinum loop or some other suitable instrument may be employed; or pus may be picked up with swabs after expression.

Smears may be prepared on microslides in the usual manner, allowed to dry and sent to the laboratory for staining and examination for spirochetes, endamebae, etc. When the secretions are scanty a drop of the patient's saliva or a drop of saline solution should be placed on the slide, the material added, and a smear prepared.

Wet preparations are very useful for examination for spirochetes and endamebae. In this case the patient must be sent to the laboratory or the examination made in

the office of the dentist. The spirochetes are readily seen stained or unstained and dark-field examination is not necessary.

Cultures of the surface of the gums are hardly worth while because of inevitable contamination with saliva. But pus expressed from pockets is quite suitable for cultures if the surface is first disinfected with 3 per cent tincture of iodine. The pus is then collected on sterile swabs and sent at once to the laboratory or directly streaked over blood agar plates and then planted in tubes of enriched broth (brain-glucose broth is recommended).

COLLECTION OF MATERIAL FROM THE EAR AND MASTOID

1. In culturing furuncles of the external auditory canal, the skin should be cleansed with alcohol and pus picked up with a small sterile swab. Plain or blood agar or Löffler's blood serum may be inoculated, as these infections are usually staphylococcic.

2. In otitis media the material is best collected by an otologist, as the external auditory canal should be cleansed, disinfected with alcohol and pus obtained on sterile swabs through a speculum and with illumination in order to guard against contamination.

3. In acute otitis media, the organism producing infection is generally obtained in pure culture; in chronic otitis media, two or more organisms are generally found.

4. The pus should be streaked on blood agar plates or inoculated in tubes of enriched broth like glucose-brain broth or glucose-hormone broth. It is a mistake to use plain agar, as this is not suitable for the cultivation of streptococci, pneumococci, etc. In acute otitis, cultures on Löffler's blood serum or slants of blood agar are sufficient, but in chronic otitis, blood agar plates should be used since the infection is generally mixed and rapidly growing organisms may readily overgrow streptococci and similar slowly growing ones. Smears on slides are also serviceable as their examination gives valuable information, especially in regard to the organisms one may expect to find in the cultures.

5. The same procedures are recommended in mastoid infections. Smears and cultures should be made at the time of operation on blood agar slants or plates or glucose-brain broth suitable for the cultivation of pneumococci and streptococci.

COLLECTION OF CEREBROSPINAL FLUID, PLEURAL AND OTHER TRANSUDATES AND EXUDATES

Cerebrospinal fluid for bacteriological examination should be collected with particular care against contamination, especially if cultures are to be made. The presence of staphylococci in cultures is rather common but of no significance unless skin contamination can be definitely excluded. As meningococci, pneumococci and streptococci in spinal fluid tend to die out rapidly, especially meningococci, *the fluid collected in sterile test tubes should be sent as soon as possible to the laboratory* where large amounts (0.5 to 1.0 c.c.) should be cultured on blood

agar, sheep serum agar or similar enriched media. Smears are also of great value and may be prepared after the cultures have been made by smearing the fluid direct if it is purulent or after securing sediment by centrifuging.

Pleural, pericardial, joint and other fluids should be collected by aspiration with a sterile syringe fitted with a sufficiently large needle and under rigid aseptic conditions with particular reference to very careful preparation of the skin. Cultures and smears are then prepared as in the case of cerebrospinal fluid.

COLLECTION OF BILE

1. The technic for collection of bile from the duodenum by nonsurgical drainage is extremely important as the bacteriological examination is almost without value unless the bile is collected with rigid precautions against the several sources of contamination. It is recommended that the method described in Chapter X, employing a special flask, be strictly followed.

2. A broth medium known to be suitable for the cultivation of streptococci should be employed; hormone broth with a pH of 7.4 to 7.6 is recommended.

3. About 20 drops of bile should be added to 150 c.c. of medium.

4. If a plating method is to be used, bile may be collected in a special sterile vial or test tube.

5. Upon delivery of the specimen, 0.5 to 1.0 c.c. of bile may be removed with a sterile pipet and plated on blood agar, the colonies being examined after twenty-four to forty-eight hours incubation. The broth method, however, is generally more satisfactory.

COLLECTION OF MATERIAL FROM FECES AND THE RECTUM

1. Feces should be passed directly into a quart-size Mason jar previously sterilized by boiling it. the rubber ring and the top for a few minutes before use. Or the patient may pass a stool into a basin previously sterilized by boiling, and a portion (especially feces with mucus) removed with a sterile spatula to a sterile wide-mouthed bottle or vial.

2. Cultures of the rectum for cholera and typhoid carriers may be made by cleansing the skin about the anus with soap, water and alcohol, followed by the introduction of a sterile cotton swab previously moistened with sterile broth or saline solution; or sterile vaselin may be applied to the anus and the finger, covered with a sterile rubber cot, inserted and swabs prepared from the cot. The swabs should be delivered promptly to the laboratory for inoculation of culture media.

3. In ulcerative colitis, cultures are best made with the aid of a sigmoidoscope or proctoscope. The ulcers should be first cleansed and material obtained with sterile swabs which should be streaked over blood agar plates or a primary culture made in an enriched broth, like brain-hormone broth for the cultivation of streptococci, etc.

COLLECTION OF BLOOD FOR CULTURES

Principles.—Since bacteria are rarely found in smears of blood, cultures are required to determine whether or not they are present. A large number of methods have been proposed and the technic employed has considerable influence upon results.

1. *Rigid aseptic technic* is required not only to guard against contamination from the skin and air in the collection of blood, but likewise in making subcultures and all subsequent examinations.

2. The methods employed may be designated as: (a) *qualitative* to determine whether or not bacteria are present; (b) *quantitative* to determine the approximate number of bacteria per cubic centimeter of blood; and (c) *massive* when large amounts of blood are cultured for the detection of small numbers of organisms, as in chronic infective types of arthritis.

3. It is helpful to know the kind of infection suspected clinically in order to choose the proper culture medium and method to be employed. It is advisable to make the culture at the peaks of pyrexial waves.

4. When relatively large numbers of bacteria are present in the blood the cultures are usually positive within 24 to 72 hours of incubation; but when only small numbers are present, the cultures may require 10 to 21 days incubation and no culture should be reported as "sterile" in less than 10 days.

5. The presence of *Staphylococcus albus*, *B. coli*, *B. proteus* and diphtheroid bacilli are frequently due to contamination and when found the culture should be repeated before concluding that they were from the blood.

6. Blood is usually collected from a vein at the elbow. In infants a superficial branch of one of the jugular veins may be employed. The internal saphenous vein as it turns over the internal malleolus of an ankle joint is also available.

Method for Collection of Blood.—Prepare the skin as follows:

1. Wash thoroughly with hot water and soap.

2. Cleanse with alcohol and sterile gauze.

3. Apply a wet dressing of 1:1000 bichloride of mercury for at least 30 minutes.

4. Apply tourniquet and request the patient to vigorously open and close the hand in order to distend the veins.

5. Light an alcohol lamp.

6. Assemble a carefully sterilized *20 c.c.* Luer syringe with No. 20 gage needle.

7. Remove gauze dressing and apply tincture of iodine over a prominent vein.

8. Ask patient to keep hand clenched. Avoid touching the skin at the site of puncture. If necessary to palpate the vein wear sterile rubber gloves or cover finger with sterile gauze.

9. Make venous puncture and **withdraw 10 to 15 c.c. of blood.** Ask patient to open hand. Release the tourniquet. Withdraw needle from the vein.

10. Inoculate the media after carefully flaming in alcohol lamp.

11. Remove iodine from the skin with alcohol. Apply flexible collodion and cotton or gauze dressing.

Routine Method for Blood Culture.—The following method can be recommended for routine cultures as it is suitable for most of the pathogenic bacteria producing bacteremia and septicemia and provides both a qualitative and quantitative culture:

1. Inoculate a flask of 150 c.c. of glucose (0.2%) hormone broth (pH 7.4 to 7.6) with 5 or preferably 10 c.c. of blood. The Kracke heart-brain broth is also recommended ·(see Chapter XVII). Other media may be used depending upon the infection suspected.

2. Place 5 c.c. of blood in a test tube carrying sterile sodium citrate and rotate thoroughly to prevent coagulation. (These tubes are prepared by placing 2 c.c. of a sterile 10% solution of sodium citrate in distilled water in each. Place in incubator or water bath until evaporated to dryness. Replace cotton with boiled rubber stoppers. Each tube will contain 0.3 gm. sodium citrate sufficient for 5 c.c. of blood.)

3. In the *laboratory* culture the citrated blood as follows: (*a*) Melt 2 tubes of plain or glucose agar in a water bath and cool to 42° C. (*b*) To one add 1 c.c. of citrated blood and to the second 2 c.c. with a sterile pipet. (*c*) Pour into 2 sterile Petri dishes and mix thoroughly; label each plate with amount of blood used. (*d*) Allow to harden and incubate covers down. (*e*) This method is better than inoculating tubes of agar and pouring plates at the bedside, as it usually permits more accurate measurement of the blood and especially if bubbles of air gain access to the syringe.

4. Incubate the flask and plates for 48 hours when a preliminary report should be made. With great care against contamination. prepare a smear of the supernatant broth and stain by the method of Gram. At the same time subculture about 0.5 c.c. of the sedimented blood and broth on a slant of blood agar. Repeat every 2 or 3 days for 10 to 21 days if there is no growth before rendering a final report. If growths develop identify the organisms. If a growth appears in the plates, report the number of colonies per cubic centimeter of blood.

Anaerobic Method for Blood Culture.—If an anaerobic culture is desired, as is advisable in culturing the blood of suspected cases of puerperal septicemia. it may be prepared by planting 1 or 2 c.c. of citrated blood at the bottom of a long narrow tube of Rosenow's brain-broth medium by means of a sterile pipet. Cover with sterile paraffin oil or vaselin. Such a culture gives partial oxygen tension or it may be incubated in a special jar in an atmosphere of hydrogen gas for more complete anerobiasis. Incubate at least 5 days before preparing smears for examination. If no growth. continue incubation and examinations for at least 3 weeks.

Method for Blood Clots.—Specimens of clotted blood submitted in sterile Keidel or test tubes for serological examinations may be utilized for preparing blood cultures after the following method by Sellers and Morris:

1. After sufficient serum has been withdrawn aseptically for serological tests, transfer the remainder of the specimen, including the clot, into the barrel of sterile Luer syringe.

2. With the nozzle turned upward, expel the air with the plunger.

3. The nozzle is then held over the mouth of the flask of broth medium and the clot "spued" into the medium by pressure on the plunger. A thorough comminution of the clot is thus obtained releasing organisms trapped in the clot.

Choice of Culture Medium.—The glucose hormone broth recommended above is suitable for the cultivation of most pathogenic organisms producing septicemia as the staphylococci, streptococci, meningococci, gonococci, typhoid bacilli, etc. For other organisms special media may be required according to the infection suspected.

Massive Methods for Blood Culture.—If the routine method described above proves sterile when but few organisms are present, larger amounts of blood may be cultured by one of the following methods:

Method of Cecil and Nicholls for Arthritis Cases.—1. Twenty c.c. of blood are taken aseptically from the arm vein of the patient, and placed in two sterile test tubes.

2. The blood is allowed to clot and placed in the ice box overnight.

3. In the morning the serum is removed and the clots transferred to bottles containing 50 c.c. of beef-heart infusion broth with a pH 7.6. The bottles are then placed in the incubator at a temperature of 37° C., and left there for one month.

4. During the month subcultures are made at five-day intervals on blood agar pour plates and in blood broth tubes. If at the end of thirty days the subcultures remain sterile, the sediment of the original bottle is examined for organisms by means of smears. Part is streaked on a blood agar plate, and part is transferred to fresh blood broth. If no organism can be demonstrated with this procedure, the blood is considered sterile.

5. All cultures and transfers are made under a hood in order to eliminate contaminations as far as possible. All contaminated cultures are discarded.

COLLECTION OF URINE

1. Urine to be examined by *culture* should always be collected by catheterization under rigid aseptic conditions.

2. If catheterization is not possible or advisable, especially in infants, the genitalia should be cleansed with soap, water, and alcohol, with special reference to the meatus, and urine collected in a sterile beaker. Special methods and apparatus have been described. It is almost impossible, however, to exclude or prevent contamination, especially with staphylococci and colon bacilli.

3. Urine to be examined for *tubercle bacilli alone* by *smear* may be collected without any special precautions other than a washing of the genitalia to prevent contamination with smegma. If guinea-pigs are to be inoculated, the urine should be passed into bottles previously sterilized by boiling and urine kept at a *low temperature* (especially important during warm weather) to prevent the multiplication of contaminating bacteria and loss of viability of small numbers of tubercle bacilli.

4. Urine to be examined for gonococci, colon bacilli, streptococci, etc., by *smear alone* may be collected in a sterile container after simple cleansing of the

parts. It should be centrifuged, however, and smears made within an hour or two after collection, or iced if a longer interval is inevitable, to prevent the multiplication of contaminating organisms.

COLLECTION OF URETHRAL AND PROSTATIC SECRETIONS

1. Smears of urethral pus are ordinarily sufficient for the bacteriological diagnosis of gonorrhea. But the method of preparing them is of practical importance. At least two smears should be prepared with cotton swabs and rolled (not rubbed) upon slides, care being taken not to pass the swab over the same surface twice. If the amount is scanty, smears the size of dimes are sufficient, as very thin smears are unsatisfactory.

2. It is sometimes advisable to furnish the patient with slides and swabs along with instructions for preparing smears of morning secretions, collected upon arising and before urination.

' 3. Smears should be allowed to dry in the air. The practice of covering a heavy wet smear with another slide is very unsatisfactory.

4. When cultures are to be made it is advisable to cleanse the meatus and secure pus by urethral massage. This should be picked up with sterile swabs immediately streaked on a suitable medium like that of Pelouze, North. blood agar, etc., or the swabs may be washed out in a small amount of sterile ascites fluid in a test tube furnished with a sterile rubber stopper and at once forwarded to the laboratory for inoculation of media.

5. The examination of urine for gonococci in chronic urethritis is not very satisfactory, although staphylococci, colon and diphtheroid bacilli, etc., from the prostate gland are readily obtained. The prostate gland should be thoroughly massaged and the *first ounce or two of urine immediately passed collected* in one or two sterile centrifuge tubes and used for examination. Or the prostate may be massaged during urination. If cultures are to be made the urine should be secured by catheterization into sterile centrifuge tubes. Whatever method is used it is important to centrifuge the urine as soon as possible at high speed and the sediment should be streaked on a suitable medium and also examined by direct smears.

6. Bacteriological examination of prostatic secretions may be conducted by having the patient empty the bladder immediately before examination. The meatus is then thoroughly washed with soap and water. While the patient constricts the urethra just behind the glans penis, the prostate is thoroughly massaged until fluid collects behind the constriction. This is then collected in one or more sterile Petri dishes and plated on blood agar. Smears may be prepared for the Gram stain at the same time.

COLLECTION OF VAGINAL SECRETIONS

1. Properly prepared smears are still of most value in the bacteriological diagnosis of gonococcus urethritis, vaginitis, bartholinitis, etc., of the female.

2. They should be prepared by rolling swabs of secretion on glass slides (not rubbed on) with care not to pass the swab twice over the same surface.

3. Pus may be secured by massage of the urethra, the Bartholin glands, and from the vagina. In adults with chronic gonorrhea, it is particularly advisable to secure secretions from on and about the cervix with the aid of a vaginal speculum. Vaginal douches should be omitted for at least several hours before examination.

4. Several smears should be prepared and properly labeled. They should be neither too thick nor too thin. If the secretions are scanty, smears the size of pennies are sufficiently large. *They should be allowed to dry in the air;* the filthy practice of covering a thick wet smear of vaginal secretion with another slide is strongly condemned.

5. Cultures may be prepared by streaking the secretions on plates of ascites agar, North gelatin agar, the Pelouze medium, or blood agar. Or swabs may be washed out into small amounts of sterile ascites fluid furnished in small test tubes with sterile rubber stoppers and the ascites emulsion sent at once to the laboratory for the preparation of plates. In medicolegal cases, fresh smears for active spermatozoa and cultures are usually required and the physician should enlist the services of the bacteriologist for aid in the technic of preparing them.

6. In infants and young children smears of the external genitalia are sometimes insufficient and unsatisfactory; especially in chronic infections with scanty secretions.

It is advisable to obtain material from the vagina by means of sterile slender cotton swabs for either smears or cultures or both. In older children, especially in those who have been under treatment, a nasal bivalve speculum may be employed as a vaginal speculum without injury, as it is particularly important to secure secretions on or about the cervix as they may show the presence of gonococci when smears of the external genitalia do not.

Vaginal washings are sometimes serviceable in these cases and may be conducted as follows:

(a) Place the child on its back with thighs spread apart.

(b) Fill the vagina with 1:4000 bichloride of mercury in normal saline solution by means of a sterile bulb or syringe.

(c) Recover the washing and transfer to a centrifuge tube. Repeat until 5 to 10 c.c. of washings have been secured.

(d) *Centrifuge as soon as possible* and prepare smears of the sediment to be stained and examined for gonococci.

COLLECTION OF MATERIAL FOR EXAMINATION FOR SPIROCHAETA PALLIDA

1. *Spirochaeta pallida* is best found by dark-field examination of fresh material. Stained smears are much less satisfactory.

2. Wet preparations for dark-field examination may be prepared in the physician's office, providing a microscopy can be done in the laboratory before motility of spirochetes is lost. Otherwise it is better to send the patient to a laboratory equipped for this work. Or the physician may collect a drop or two of secretion in a Wright capillary blood tube. The ends should be sealed with wax or paraffin

(not with heat). In the laboratory preparations are made for dark-field examination.

3. *Spirochaeta pallida* may be found in chancres (genital and extragenital), mucous patches, condylomata and in some skin lesions as well as in swollen lymph glands, although examinations for the organisms are practically confined to sores regarded as possible primary lesions or chancres.

The examination of lesions on the lips is quite reliable but when occurring within the mouth great care is required, since *Spirochaeta microdentium* of the saliva is morphologically indistinguishable from *Spirochaeta pallida*.

4. Surface exudates should not be used, as the spirochetes are usually in the tissues. An effort should be made to secure tissue "juice" with as little blood as
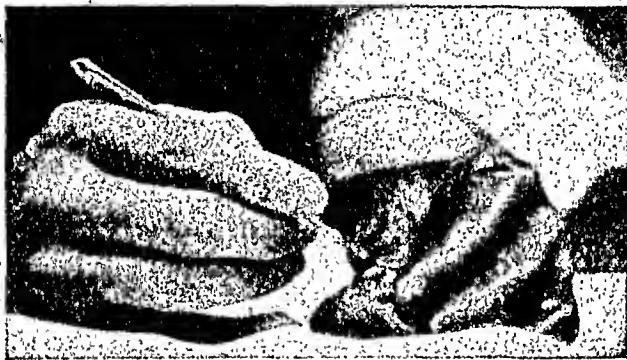
FIG. 193.—METHOD OF SECURING CHANCRE MATERIAL
(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

possible. All applications of antiseptics should be omitted for at least several hours before the examination is made.

5. Thin glass slides (free of scratches) and cover glasses are required; also sterile saline solution and usually an instrument like a scalpel or stiff platinum wire loop for securing tissue juice. Capillary pipets may be used for collection. At least two slides should be prepared. Place a drop of saline solution on each.

6. The lesion should be grasped between the thumb and forefinger (protected with rubber gloves or gauze) and squeezed (Fig. 193) to secure tissue juice which may be transferred to the saline on the slides with a sterilized platinum wire or loop. If this is not successful, the lesion may be gently scraped (while being squeezed to prevent bleeding); the material transferred to slides and mixed with the saline solution. A cover glass should be applied (being careful to avoid floating) and the dark-field examination made at once. Or smears may be made and allowed to dry in the air if a staining method (like that of Fontana) is to be employed. If the sore is quite painful, one may first apply a few crystals of cocaine or a few drops of novocaine solution to anesthetize it.

7. Lymph gland material may be obtained by puncture, using a sterile 1 c.c. syringe fitted with a No. 22 needle and injecting 0.5 to 1.0 c.c. of sterile saline

solution followed by aspiration and the preparation of slides with a few drops of the fluid.

COLLECTION OF MATERIAL FOR EXAMINATION FOR B. DUCREY (CHANCROID)

1. Pus may be obtained by aspiration of a bubo with syringe and needle and inoculated into a medium of two parts agar mixed with one part of sterile human, dog, or rabbit blood; smears should be prepared at the same time.

2. An open ulcer may be painted with tincture of iodine and covered with sterile gauze; 24 hours later prepare smears and cultures of pus collecting under the dressing.

COLLECTION OF MATERIAL FOR EXAMINATION FOR LEPROSY

1. Lepra bacilli are usually present in the tissues of leprous lesions in large numbers, especially in tubercular leprosy. They may also be found in the nose and fauces.

2. The bacilli are chiefly in the fixed tissue cells, and it is usually necessary to
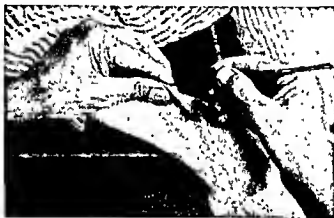
FIG. 194.—SECURING MATERIAL FROM A LEPROUS LESION

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

scrape the lesions for proper material; little or no pain is produced (Fig. 194).

3. Place a drop of saline solution on each of several slides. Grasp the lesion and, while squeezing to prevent bleeding, scrape with a scalpel or a safety-razor blade and transfer the scraped-up tissue to the slides. Make spreads and allow them to dry in the air, to be followed by staining for acid-fast bacilli.

4. Secure material from the nose and fauces with sterile cotton swabs and prepare smears in the usual manner.

COLLECTION OF MATERIAL FROM WOUNDS

1. Smears and cultures are required. It is recommended that purulent secretions be collected in sterile tubes with the aid of sterile swabs and sent to the laboratory.

If gangrene infection is suspected, a statement to this effect should accompany the specimen so that special anaerobic methods may be employed for *B. welchii*, *Vibrion septique*, etc.

2. If cultures are made at the bedside, blood agar plates are recommended, especially if mixed infection is suspected. Otherwise blood agar slants on glucose-hormone broth may be used, suitable for the cultivation of streptococci. Plain agar may be used only in case of simple abscesses.

3. If infected wounds are being irrigated according to the Carrel methods, it is necessary to interrupt irrigation for at least two hours. Smears are made on glass slides with a stiff platinum loop or sterile cotton swabs. The areas should be selected with great care with particular reference to the deepest parts, necrosed points of fascia, the surface of damaged bone and culdesacs where secretions can accumulate protected from the irrigation solution. Bleeding areas should be avoided.

4. The secretions thus collected are spread out on slides which should be properly labeled with the name of the patient and the region of the wound whence the secretion was taken. They should be allowed to dry before delivery to the laboratory.

COLLECTION OF NECROPSY MATERIAL

1. When bacteriological examinations are to be made in the course of a necropsy, it is imperative to remove the material at the earliest possible time after death and at least within an hour or two in order to avoid the increase of secondary invaders and the postmortem invasion of the tissues with intestinal bacteria.

2. The body should not be embalmed if cultures are to be made, although smears and examinations for bacteria in sections of tissues may be made.

3. An area of the heart, liver or other organ or tissue to be cultured should be first seared with a cautery iron and then opened with a cautery or sterile knife and material obtained with a sterile pipet, cotton-swab, stiff platinum wire or scalpel. Blocks of tissue may be removed, dipped into boiling water for surface sterilization and then cut into bits under aseptic precautions for cultivation in brain-hormone broth or similar enriched media.

DISPOSAL OF MATERIAL SUBMITTED

1. It is a good practice to retain specimens of pus, various secretions, spinal and other fluids, etc., for several days at least in case it is necessary or advisable to repeat the examinations. Cultures should be retained for a week or longer in case confirmatory tests are desired. It is also advisable to retain important smears properly labeled.

2. All specimens containing or likely to contain pathogenic organisms should be placed in a pail or pan and the latter autoclaved at the close of the day. Paper sputum cups should be burned.

3. It is a good practice to add a few c.c. of 5 per cent phenol or tricresol to all discarded cultures (replacing the stoppers), including Petri plates.

4. Pipets used for handling infectious material should be placed in a jar, crock, or pan of water containing phenol or tricresol for disinfection.

5. Boil all instruments and syringes immediately after use.

6. Wash the work table routinely with 10% crude cresol or some other suitable antiseptic.

CHAPTER XVI

METHODS FOR THE PREPARATION AND STERILIZATION OF GLASSWARE

Principles.—Test tubes, Petri dishes, and flasks should be made of good quality glass in order to (1) withstand repeated steam sterilization at 121° C. (approximately 15 pounds pressure) and hot air at 170° C. with the minimum of decomposition and (2) to contain the smallest amounts of free alkali so that there will be the minimum difference between the initial and final reactions of culture media. Strain-tested Pyrex and Nonsol glass are recommended because of their stability toward distilled water, and low coefficient of expansive and mechanical strength.

SELECTION OF GLASSWARE

1. *Test tubes* should be of thicker walls than are used for chemical work, without lips in order to facilitate plugging and storage, and of such size as to fit in the wire test-tube racks now in common use. Three sizes are usually sufficient: 100 by 13 millimeters for slants and broth, as 5 c.c. in each is sufficient and economical; 200 by 13 millimeters for giving a high column of medium with varying degrees of oxygen tension (low at the bottom and high at the top); 120 by 16 millimeters for carrying to 10 c.c. or larger amounts of agar, gelatin, etc., for pouring Petri plates and for holding sterile swabs.

2. *Petri dishes* subjected to steam pressure sterilization should be of alkali-free glass and capable of standing repeated sterilization without corrosion (N-101-AF glass is recommended).

FIG. 195.—FERMENTATION TUBE SUPPORT   FIG. 196.—WIDE MOUTH RECTANGULAR BOTTLE

Where hot air sterilization only is used, dishes of selected lime glass are sufficient, providing they are thoroughly dried before being placed in the sterilizer. Covers of white Coors porcelain are recommended: unglazed inside for providing sufficient absorbing surface for water of condensation, and glazed outside and at the sides for facilitating the removal of pencil markings. The 10-centimeter size is ordinarily employed.

3. *Erlenmeyer flasks* of Pyrex or Nonsol glass with vial mouths are recommended and three or four sizes are usually sufficient: 300 c.c. capacity for blood cultures and 500 to 3000 c.c. capacities for storage of culture media, etc.

4. *Smith* fermentation tubes (Fig. 195).

5. *Blake* bottles (Fig. 196) and *Kolle flasks* are commonly employed for mass cultures in the preparation of stock vaccines, bacterial antigens, etc.

6. *Microslides* should be of noncorrosive hard glass with grounded edges, free of scratches and of the usual 3 by 1 inch size with a thickness of 1 to 2 millimeters. For dark-field work the slides should be carefully selected, free of scratches and *within 1.45 to 1.55 millimeters in thickness.*

7. *Micro coverglasses* should be of noncorrosive glass; soft white glass should not be used. Squares (22 millimeters), rectangles (22 by 36 millimeters), and round glasses (15 millimeters) may be recommended for routine work.

CLEANING GLASSWARE

Used Glassware.—1. Glassware should be washed as soon after use as possible.

2. If glassware is contaminated, it should be autoclaved before being washed. After removing from the autoclave and while still warm, remove all cotton plugs and empty.

3. Place the glassware in warm water, add enough good quality soap to make plenty of suds and scrub well with a brush.

4. Rinse well in running water, preferably, rinsing once in tap water and twice in distilled water. Allow the glassware to drain and dry.

5. Tubes with paraffined stoppers should be *separately* cleaned because soiling with melted paraffin renders cleaning more difficult. After autoclaving and while still hot, remove the cotton stoppers, empty and immerse the paraffin-soiled glassware in hot water to which is added some in fine shavings to make about a 5% solution of the soap. Boil slowly for an hour, allow to cool somewhat and scrub well. If the paraffin is not removed, boil again in fresh soap solution, scrub well, rinse and allow to drain and dry.

Pipets should be placed, *immediately after using*, in a tall crock or cylinder full of 2 per cent lysol solution (for disinfection) with a layer of cotton on the bottom. To wash pipets, hold them in flowing tap water, or better, use a water suction pump attached to the faucet. Place the mouth end of the pipet in the rubber tube connected with the pump and the other end in a container of tap water and suck water through for about one-quarter minute; rinse by draining distilled water through the pipet, put aside to drain and dry.

Slides and coverglasses may be placed in 2% lysol solution which in a week or so loosens Canada balsam; this solution also disinfects hanging drop and dark-field preparations.

1. Boil in soapy water or 10% solution of chromic acid for ten minutes.

2. Rinse in running water, drain and polish or keep the slides and cover glasses in 70% alcohol.

New Glassware.—New test tubes, flasks, Petri dishes, pipets and other glassware are likely to contain free alkali and should, therefore, be allowed to stand for a few hours in 2% hydrochloric acid. Rinse well with tap water, then treat as directed in steps 3 and 4 under used glassware.

Slides and coverglasses may be cleaned with alcohol and used directly or stored in 70% alcohol until needed.

Cloudy Glassware.—When glassware (test tubes, Petri dishes, pipets, slides, coverglasses, etc.) retains cloudiness that cannot be removed by means of washing with soap, use potassium bichromate sulphuric acid cleaning solution, prepared as follows:

Commercial potassium bichromate .... 60 gm.
Tap water .... 300 c.c.
     Dissolve with the aid of heat, cool and add
     slowly with constant stirring:
Commercial sulphuric acid .... 460 c.c.

Fill test tubes, beakers, and flasks with cleaning solution and place pipets, slides, coverglasses, etc., in pans full of the solution and allow to remain twenty-four hours. To be followed by *thorough* rinsing in running water to remove all traces of cleaning fluid.

The fluid can be used repeatedly and when it appears to lose its strength more potassium bichromate and sulphuric acid should be added. It is, however, very corrosive and should *not* be used more frequently than necessary.

PLUGGING TEST TUBES, FLASKS AND PIPETS

1. Test tubes may be plugged as follows: Using a good quality, long-fiber cotton batting which is nonabsorbent, cut strips 1½ to 2 inches in width; unfold the strips so that they are uniformly one layer thick; dip one end of a glass rod (about 3 inches long and 3 mm. in diameter) in water, then proceed to roll the flat strip of cotton about the moist glass rod until a plug of sufficient size is obtained; tear plug away from the remainder of the cotton, rolling a few times in the fingers and insert into a test tube. Remove the glass rod from the center of the cotton plug by turning a half turn in the opposite direction and withdrawing. The above method gives a well-formed plug which will not flatten out when withdrawn and reinserted into the test tube. The cotton plugs for test tubes should be large and firm enough to exclude dust and germs, should project sufficiently beyond the tube for handling and be tight enough to permit the test tube to be lifted by the plug.

2. In the case of flasks, the strips of cotton should be cut wider and may be rolled with the fingers to the proper size.

3. In case it is desired to protect the interior of test tubes, flasks or other vessels from cotton fibers, a square of gauze or cheese cloth may be placed over the mouth of each vessel before inserting the cotton plug. This also allows support to the cotton plug in the case of flasks with large openings which require a large plug.

4. Added protection against contamination may be had by placing a piece of wrapping paper secured around the neck of the flask by means of cord.

5. The mouth ends of pipets should be plugged with a small pledget of cotton

to protect the worker against accidental contamination of the mouth while pipeting micro-organisms. The small bit of cotton may be inserted into the bore of the pipet by means of a piece of wire or a hair pin. The cotton should not extend beyond the end of the pipet in such a way as to prevent placing the finger firmly against the end. Any loose fibers can be burnt off by passing the ends of the pipets through a gas flame.

STERILIZING GLASSWARE

1. Test tubes, Petri dishes, pipets and flasks should be *perfectly dry* before hot air sterilization (Fig. 197), to prevent breakage and reduce decomposition. Do not use moist heat.

2. Petri dishes and pipets may be wrapped singly or in multiples with paper. Or they may be placed in special cans of copper or sheet iron for sterilization (Fig. 198).

3. Place in hot air sterilizer and *gradually* raise the temperature to about 170° C., which is sufficient for turning cotton and paper to a faint yellow color.

4. Avoid overheating to prevent charring of cotton and paper and the release of oils
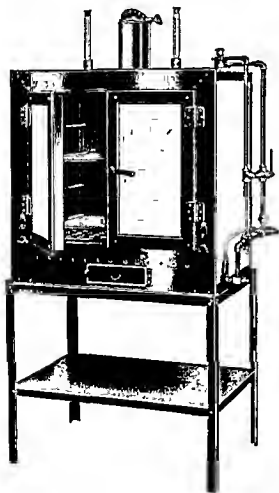
Fig. 197.—Hot Air Sterilizer

Fig. 198.—Pipet Box

from cotton. Do not allow cotton plugs to touch the walls of the oven.

5. Heat for an hour at 170° C., and then allow the sterilizer to cool to at least 60° C. before opening the door, to prevent cracking by too sudden contraction of the glass.

6. While it is not imperative to sterilize test tubes and flasks before filling them with culture media, it is advisable to do so for ensuring better sterilization and for the purpose of molding the plugs for easier removal during bacteriological work.

7. When large amounts of glassware are to be sterilized, the use of a good thermo-regulating valve on the sterilizer together with a recording thermometer are recommended.

CHAPTER XVII

METHODS FOR THE PREPARATION OF CULTURE MEDIA

Principles.—1. Culture media are artificial foods for bacteria, containing soluble albumins, carbohydrates and other organic compounds as well as water and salts. Native proteins are probably not directly utilized and peptone is added to supply available nitrogen. Vitamin-like substances as well as other accessory substances present in blood, serum, ascitic fluid and fresh vegetable extracts help the growth of all pathogenic bacteria, especially streptococci, pneumococci, gonococci, *B. pertussis*, etc. Certain organisms such as the hemoglobinophilic *B. influenzae* require both the thermolabile V and the thermostable X factors in blood and vegetable tissues. Carbohydrates and especially glucose may be required. Dyes may be added either as indicators of metabolic activity or because of their selective inhibitory action on some bacteria, thereby aiding in isolation of others (notably the colon-typhoid group).

2. Therefore a very large number of media have been described for meeting special requirements. Practically all have as a general basis an infusion or watery extract of meat (usually beef or veal).

3. Heating for sterilization and filtering for clearing affects meat infusion deleteriously, and lost nutritive material is replaced in part by the addition of peptone, which is water-soluble and not precipitated by boiling; also by adding blood, serum, etc. Beef extract is generally inferior to fresh meat infusion because of the loss of nutritive substances due to prolonged heating in manufacture. It is, however, useful for the cultivation of the more hardy organisms and especially convenient for small laboratories.

4. The method of sterilization of culture media is, therefore, an important matter. Autoclaving may destroy nutritive principles. Fractional sterilization in an Arnold sterilizer is generally to be preferred.

5. Most pathogenic bacteria are quite susceptible to acids and alkalis and grow best in media near neutrality or slightly alkaline or acid to it. The meat bases are more or less acid and usually require the addition of an alkali (sodium hydroxide). Culture media tend to become more acid during sterilization because of hydrolysis of some of the constituents. On the other hand, they may become more alkaline because of alkali derived from glass, especially if cheap, soluble glass containers are employed.

6. Culture media may be (*a*) fluid (broth, milk, peptone water, etc.); (*b*) solid (agar, gelatin, coagulated serum or egg, etc.), or (*c*) semisolid (broth with small amounts of agar, gelatin or coagulated serum); the last are especially useful for carrying stock cultures.

7. The most important principles involved in their preparation are: (a) To secure the maximum amounts of growth-stimulating substances. and (b) for this purpose to adjust the final reaction to the optimum pH for the organisms to be cultivated; (c) to use the minimum degree of filtration; (d) to use the minimum amount of heat for sterilization.

DEHYDRATED OR POWDERED CULTURE MEDIA

Dehydrated culture media have been marketed for a number of years by the Digestive Ferments Company (Difco) in Detroit, Michigan. More recently, the Baltimore Biological Laboratory, Baltimore, Maryland, has also undertaken to manufacture such powdered media. These types of media are often very satisfactory and are especially to be recommended to small laboratories where a trained media-maker is not available. In larger laboratories the cost of such prepared media often becomes considerably higher than home-made media. For new methods of cultivation, isolation, or description of bacteria, only media whose composition and method of preparation are fully known or described should be used. Prepared powdered media may be used for routine work only when they can be shown to behave similarly to the original media described for the purpose.

BASIC CONSTITUENTS OF CULTURE MEDIA

Peptones.—Peptones are peptic or tryptic digests of proteins. Most peptones in general use are prepared commercially and the exact methods of their preparation are kept secret. At present there are sold in the United States some 15 to 20 different kinds of peptone. Most of these are either made from casein or from beef. Many of them seem to be much alike and it is safe to say that more than half of them could be eliminated to the great advantage of the bacteriological profession. The present peptone situation is unfortunate from a scientific standpoint. There is no necessity for having one of the most important ingredients of culture media of unknown composition and mode of preparation, rendering much bacteriological work of doubtful permanent or fundamental value. A classic example is Sabouraud Agar which now cannot be duplicated. If Sabouraud had given exact and detailed directions for the preparation of the peptone he used, his classical work on fungi would now be of much greater value than it is. Media are constantly appearing in the literature in which not only peptone but many other proprietary products are specified.

Unfortunately sufficiently fundamental and detailed work does not seem to have been made on the preparation of peptone to enable the bacteriologist to tell the manufacturer exactly how he wants his peptone made, or to enable him to make the best peptones for himself. A number of publications have appeared giving directions for the preparation of peptone, but none of these have been extensive enough to command the attention of bacteriologists in general. Peptones may be made from various proteins such as casein or various animal organs. *Peptic digests* are usually made by adding minced animal stomachs to the ground protein in an acid medium. The temperature is generally kept at about 50° C. and the process

of digestion controlled by making tests from time to time for various amino acids. The biuret test and the test for tryptophane are commonly used. *Tryptic digests* are made in slightly alkaline solution using pancreatic extract as a source of trypsin. If the digestion is carried out for more than 5-6 hours some preservative such as chloroform or toluol must be added.

It is not always easy to decide what peptone to use for a particular purpose. Generally a considerable amount of comparative work must be done. In any formula for a bacteriological culture medium a statement should always be made as to what kinds of peptone were tested and which ones were found to be most suitable.

Meat Extract.—Commercial meat extract is generally made from so-called "soup liquor" obtained from the pre-cooking of meat intended for canning. The meat for canning is placed in large iron baskets and suspended in tanks of cold water. The whole is then heated with steam for 30-40 minutes. The liquor from this process is evaporated on vacuum pans at a temperature around 160° F. for some 4 hours. The concentrated extract is further evaporated in a kettle for some 8 hours or until the water content is down to about 22%. During this process the extract turns dark brown and all the sugar and gelatin in the original juice are destroyed. The exact processes used by various companies are more or less commercial secrets.

Meat Infusion.—Meat infusion forms the basis of most of the richer bacteriological culture media. Such infusions are most commonly made from the muscle of beef, veal or pork, or from beef heart. The meat used should be absolutely fresh and is preferably bought directly from the abattoir. Autolysis of the meat during storage seems to develop toxic substances and inferior infusions are obtained from such meat. There are several methods in general use for making meat infusion. For all these methods, only fresh lean meat is used, which is ground in a meat chopper. Distilled water is added to the meat in the proportion of 2 parts of water to one of meat and the whole generally allowed to infuse overnight. To minimize bacterial growth and autolysis the water which is added to the meat should be very cold, preferably refrigerator temperature. The next day the mixture of meat and water is generally brought to a boil and the meat strained off. Some prefer to separate the meat from the liquid part while still cold and not allow the meat to be cooked with the infusion. After the coagulated meat is strained off the reaction is adjusted by adding alkali to about pH 8. The infusion is then cooked for a few minutes and filtered through paper.

Recommended Procedure for Making Meat Infusion.—Fresh, lean meat is ground and thoroughly mixed with 3 times its weight of distilled water. This is allowed to infuse at room temperature for 1 hour and then the whole mixture is cooked for 5 minutes. The cooked meat is removed by straining and the infusion filtered through paper. The reaction is then adjusted to about pH 8, the infusion cooked for 10 minutes, and filtered through paper. The volume is then restored to equal that of water originally added.

There is a mistaken notion among many bacteriologists that "double strength" infusion can be made by using twice the usual amount of meat to the same amount

of water. To illustrate the amount of "solids" obtained from various proportions of meat and water, and from allowing the meat to infuse for various lengths of time, the following table shows the amount of alkali necessary to bring pH to 7.5, and the amount of solids in infusions made with various ratios of meat and water. The ground meat was infused 1 hour and then cooked 5 minutes. Fresh pork was used.

TABLE I

	Amount of Water to 100 Grams of Pork			
	100	200	300	600
c.c. of N/10 NaOH to 100 c.c. of infusion.....	22	14	10.5	5.5
c.c. of N/10 NaOH to total infusion.........	22	28	31.5	33
% of solids in the infusion.................	2.12	1.29	0.975	0.51
Weight of total solids in the infusion........	2.12	2.58	2.925	3.06

In the following table is shown a comparison of the amount of alkali to bring the pH to 7.5, and the amounts of solids in 2 infusions prepared by different methods: 2 parts of water was added to 1 part of pork. infused 24 hours in the refrigerator. One infusion was made by squeezing the juice from the meat, and the other by cooking the meat with the water.

TABLE II

	Squeeze Method	Cook Method
c.c. of N/10 NaOH to 100 c.c. of infusion........	12	15.5
c.c. of N/10 NaOH to total infusion.............	24	31
% of solids in the infusion.....................	0.89	1.44
Weight of total solids in the infusion...........	1.78	2.88

SUGAR-FREE INFUSION (FERMENTED INFUSION).—As a base for fermentation studies with fastidious bacteria the meat infusion, not yet titrated and still acid. is generally inoculated with a culture of colon bacilli or yeast and incubated for 15-18 hours. A better method seems to be to add a large amount of yeast in the form of baker's or brewer's yeast and allow the fermentation to proceed for only 2 hours. By this method considerably less autolysis or proteolysis takes place.

Gelatin.—Gelatin is a protein obtained from bones, hornpiths, and hidestock. The bones are not used raw but are first subjected to a chemical process which produces what is known as osseine, which is the organic substance of bones. The raw bones are washed, crushed, and then degreased by means of benzine or carbon tetrachloride. The mineral substances of the bones (mostly phosphate) are then extracted by diluted acid, usually hydrochloric or sulphurous, or a combination of these two. A valuable by-product, acid phosphate. is thus produced. The material left after extraction of the fat and mineral substances is called *osseine*. The osseine is subjected to various treatments of washing. alkali treatments to destroy the last

traces of fat, more washing, acid treatment to destroy the alkali, and finally washing to obtain a neutral product. The material is then placed in extraction tanks where the gelatin is extracted by means of warm water (130-200° F.). The thin gelatin solution (4-6%) is drawn off from the extractor and clarified by filtration and settling. For making sheet gelatin the dilute solution is spread on drums or endless belts on which it is chilled, scraped off and placed on cotton or wire netting to dry. For making powdered gelatin the solution is generally concentrated to 10-20% and hardened in molds. It is then cut into thin pieces and dried on cotton or wire netting. The sheets or pieces of gelatin are dried by warm air, generally at from 85° to 105° F. There is some variation in gelatin made by different processes. The most important from a bacteriological standpoint is the geling strength and clarity. The temperature and strength of acid used in the manufacture is probably responsible for any loss of geling strength. Gelatin media should not be heated more than is necessary.

Agar-Agar.—Agar-agar is obtained from certain types of "seaweed" found along the Pacific coasts. The greatest part of the agar supply comes from Japan, while a limited quantity is produced in California. The agar is extracted from the seaweed by boiling water. The resulting agar solution is allowed to gel, cut into strips and frozen. The frozen strips of agar are then allowed to thaw out, the agar dried, and bleached in the sun. Much agar is sold for bacteriological purposes in the original strip form cut into 8-10 inch lengths. The quality of agar used by bacteriologists has undergone considerable improvement during the last few years. It is now possible to obtain agar which is ground to any desired fineness and which gives a perfectly clear solution in water. Powdered agar needs no preliminary soaking in cold water and goes into solution after only 1-2 minutes of cooking.

For making streak plates concentrations of agar from 1.5 to 2% should be used. For pour plates the best concentration is 1.2 to 1.5%. Semisolid agar media generally contains about 0.3% agar.

Due to the greater ease with which it goes into solution and the saving in time and effort by eliminating the necessity for filtration it is recommended that only powdered agar giving a clear solution in water be used. This is best obtained from manufacturers of bacteriological media such as the Digestive Ferments Company or the Baltimore Biological Laboratory.

DETERMINING AND ADJUSTING THE REACTION OF CULTURE MEDIA

Electrometric Methods of Determining pH of Media.—Electrometric methods are the most fundamental methods of determining the reaction and for the titration of culture media. All colorimetric methods must be standardized electrometrically. While electrometric methods are more fundamental and accurate they also require greater care, more controls, and are generally too cumbersome for the small laboratory to use. This is especially true of the most fundamental method of all which is the use of the hydrogen electrode. By means of this electrode a direct measurement is made of the potential, or effective concentration, of hydrogen ions.

All other methods, whether electrometric or colorimetric must be standardized against the hydrogen electrode.

The glass electrode and the quinhydrone electrode are simpler to use than the hydrogen electrode but have a limited usefulness. For titrating or determining the reaction of highly colored liquids, checking on colorimetric methods, etc., these methods are useful and sometimes indispensable.

For a detailed discussion of these methods the reader is referred to such texts as Clark's Determination of Hydrogen Ions. For the average bacteriological laboratory the colorimetric methods are sufficiently accurate and to be preferred.

Method of Adjusting pH.—In the pH scale acid reactions scale lower than 7 and alkaline reactions scale above 7.

The pH of culture media is not increased or decreased in direct proportion to the amount of acid or alkali added. This is due to the action of buffer substances present in the media.

Buffers are substances which tend to inhibit a change in the pH when acid or alkali is added to a solution. Such substances as peptone, meat extracts, and phosphates have this inhibitory quality. Knowing the pH of a solution, it is not possible to figure mathematically the exact amount of acid or alkali to add to obtain a certain lower or higher pH, because of the action of these buffers.

Indicators are substances which when added to a solution assume a definite color at a particular pH. One indicator is useful only for a certain limited range of the pH scale. This is from the pH at which color begins to change to the pH where the color change is complete. *e.g.*, phenol red gives a yellow color in solutions with low pH (acid side): at a pH 6.8 it begins to change to a pale pink which is intensified until a red is assumed at a pH 8.4 (alkaline side) and above.

It is obvious that several indicators are required to cover the entire pH range. But for the adjustment of culture media only two or three are required to cover the pH range usually employed.

Color standards of the indicator can be prepared in standard buffer solutions to represent the shade of color obtained with a particular indicator at various points in the pH scale. However, these are difficult to prepare as each buffer solution, which is usually a mixture of some acid and its alkali salt, should be very carefully tested by the electrometric method for checking the exact pH.

Standard phosphate solutions (Sørensen) may be prepared as follows:

1. Prepare a M/15 solution of disodium phosphate (Sørensen)[1] in ammonia-free distilled water.

2. Prepare a M/15 solution of monopotassium phosphate (Sørensen)[2] in ammonia-free distilled water.

3. Mix the two solutions in various proportions, measuring with extreme accuracy:[3]

[1,2] These salts should be specially prepared for this purpose. They can be obtained from reliable drug houses under the name of Sorensen's potassium phosphate and sodium phosphate.
[3] These mixtures are not stable and remain good for but two to three weeks.

M/15 Sodium Phosphate, c.c.		M/15 Potassium Phosphate, c.c.		pH
97.5	+	2.5	=	8.3
95.0	+	5.0	=	8.0
92.0	+	8.0	=	7.8
88.0	+	12.0	=	7.6
82.0	+	18.0	=	7.4
73.0	+	27.0	=	7.2
62.0	+	38.0	=	7.0
50.0	+	50.0	=	6.8
37.0	+	63.0	=	6.6
26.0	+	74.0	=	6.4
18.0	+	82.0	=	6.2
12.0	+	88.0	=	6.0

4. Unless the laboratory is especially equipped for preparing and testing the standard solutions, it is advisable to purchase them. Standard sets covering the range of any of the indicators used in adjusting culture media can be purchased from laboratory supply houses

The indicators most commonly used for bacteriological work are:

Methyl red	= red to yellow	= pH 4.4 to 6.0
Bromcresol purple	= yellow to purple	= pH 5.2 to 6.8
Bromthymol blue	= yellow to blue	= pH 6.0 to 7.6
Phenol red	= yellow to red	= pH 6.8 to 8.4

The bromcresol purple and bromthymol blue are used in 0.04 per cent; phenol red and methyl red in 0.02 per cent solutions in 95 per cent alcohol.

Colorimetric method using Buffer Standard.—1. Materials required: (a) freshly distilled water (ammonia free); (b) test tubes; (c) comparators block (Fig. 199); (d) several 10 c.c. pipets; (e) buret; (f) standard color tubes of the desired pH range (Fig. 200).

2. Place 2 c.c. of the medium to be tested and adjusted in each of two test tubes. Add 8 c.c. of freshly distilled water to each. If the water has been exposed to air for some time before use or is not freshly distilled, it should be boiled and allowed to cool to below 40° C. just before use. If agar is to be tested it can be measured while liquid and diluted with warm water (not over 40° C.) to prevent solidification.

3. To one of the tubes containing diluted medium, add the same amount of indicator as used in the standard tubes (ordinarily 0.25 to 0.5 c.c.). Place the tube in the right front hole of the comparator block.

4. The other tube of diluted medium is placed in the left front hole of the comparator block.

5. Fill a third tube with distilled water and place in the right back hole of the comparator block, which is therefore behind the tube containing medium plus indicator.

6. From the set of standard color tubes select one which appears to match the tube of medium plus indicator. Place it in the left back hole, which is in back of the tube containing medium only. Compare the colors by viewing through the

FIG. 199.—CLARK
COLOR COMPARATOR
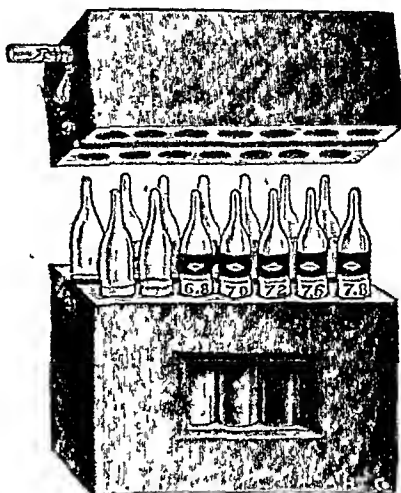
FIG. 200.—LAMOTTE HYDROGEN ION
SET

observation holes of the comparator block with either a daylight lamp or window furnishing light from the back of the block.

If the colors do not match, select another standard tube either lighter or more intense in color as may be indicated, and again examine.

Repeat this procedure until the tube is found which matches. The pH value marked on the tube which matches indicates the pH of medium.

7. To change the pH of a medium to another point in the pH scale, place the standard tube marked with the pH desired in the left-hand back hole and allow the other three tubes to remain in the same positions.

8. Slowly and carefully add N/20 solution of sodium hydroxide drop by drop to the tube containing medium and indicator until the color matches the color standard tube of the desired pH. Note the amount of N/20 solution used. The addition should be made from a buret or pipet and carefully measured. It is necessary to mix at intervals during the addition of the sodium hydroxide, especially when approaching the end-point, in order to avoid adding an excess.

9. To adjust the bulk of medium it is necessary to add to it sodium hydroxide in the same proportion as used to adjust the sample tested.

Suppose there are 2000 c.c. of medium to be adjusted to a pH 7.6, and that it required 0.3 of N/20 sodium hydroxide to bring the tube containing 2 c.c. medium and indicator to the same color as the standard tube marked pH 7.6. It would there-

fore require 300 c.c. of N/20 sodium hydroxide to adjust 2000 c.c. of medium. But the addition of this amount of N/20 would increase the volume too much, so it is better to add one-twentieth of this amount or 15 c.c. of a N/1 solution of sodium hydroxide.

The following is a simple method for calculation. Let A equal the amount of

Fig. 201.—HELLIGE COLORIMETER

N/20 required to adjust 2 c.c. of the medium to the proper pH, and B the number of c.c. in the bulk of medium to be adjusted:

$$\frac{A \times B}{40} = \text{c.c. of N/1 sodium hydroxide to add to bulk of medium}$$

or

$$A \times 25 = \text{c.c. of N/1 sodium hydroxide to add to each liter of medium}$$

Colored Glass Standards.—A convenient type of pH colorimeter for large laboratories is the Hellige model (Fig. 201) containing permanent color standards as glass disks. The proper disk is inserted in the box and 10 c.c. of medium added to the tubes, one of which contains the indicator. A wide range of pH values may be determined rapidly by using the appropriate disk. A standard solution of indicator must be used.

Gillespie Standards.—The method of Gillespie is perhaps the most reliable of the various colorimetric methods in that no standard solutions of any kind are required. The color corresponding to any desired pH is obtained by using a set of two tubes, one of which has 20 c.c. of dilute acid and the other 20 c.c. of dilute alkali. A certain number of drops of indicator is added to the acid and alkaline tubes. The ratio of the amount of indicator in the acid and alkaline tubes deter-

mines the shade of color and hence the pH, when looking through both tubes. The indicator solution need not be of any exact concentration since the same indicator is added to both the standard and the unknown. In Table III is given the respective number of drops of various indicators to be added to the acid and alkaline tubes to obtain any desired pH. In this table is shown the pH obtained when various amounts of indicator is added to the alkaline and acid tubes.

TABLE III

Drop-Ratio Alkali: Acid	pH M.R.	pH B.C.P.	pH B.T.B.	pH P.R.	pH Thymol Blue
2——18	4.05	⁻5.30	6.15	6.75	7.85
3——17	4.25	⁻5.50	6.35	6.95	8.02
4——16	4.40	5.70	6.50	7.10	8.20
5——15	4.50	5.80	6.60	7.20	8.30
6——14	4.60	5.90	6.70	7.30	8.40
7——13	4.70	6.00	6.80	7.40	8.50
8——12	4.80	6.10	6.90	7.50	8.60
9——11	4.90	6.20	7.00	7.60	8.70
10——10	5.00	6.30	7.10	7.70	8.80
11—— 9	5.10	6.40	7.20	7.80	8.90
12—— 8	5.20	6.50	7.30	7.90	9.00
13—— 7	5.30	6.60	7.40	8.00	9.10
14—— 6	5.40	6.70	7.50	8.10	9.20
15—— 5	5.50	6.80	7.60	8.20·	9.30
16—— 4	5.60	6.90	7.70	8.30	9.40
17—— 3	5.75	7.00	7.85	8.45	9.50
18—— 2	5.95	7.20	8.05	8.65	9.75

A comparator block with two sets of three holes in series is used. These are sold by laboratory supply houses as the Gillespie Comparator Block. Large tubes of uniform diameter should be selected for use and graduated to 10 and 20 c.c. for convenience. Only 4-5 drops of N/10 acid or alkali should be added to the standard tubes since an excess of acid or alkali may change the color of the indicator. The medium to be titrated is diluted as desired and 10 c.c. placed in each of two tubes. To one of these is added 10 drops of the indicator and this is placed in the block in series with two tubes of water. The tube of medium without indicator is placed in series with the color standard.

Titrimetric Phenolphthalein Method.—The color changes of phenolphthalein are:

(a) Colorless: medium is acid.

(b) Faint pink: medium is neutral to phenolphthalein but actually on the alkaline side of true neutrality equal to pH 8.2 to pH 10.0.

(c) Red: medium is alkaline.

The plus sign is used for denoting acidity and the minus sign for alkalinity, according to Fuller's method.

1. The materials required are (Fig. 202):

    (a) A buret (B) held in a clamp on a ring stand (A)

    (b) Casserole (C)

(c) Glass stirring rod

(d) Normal (N/1) and twentieth normal (N/20) sodium hydroxide solutions

(e) Indicator: dissolve 0.5 gram phenolphthalein in 100 c.c. of 50 per cent alcohol (0.5 per cent solution)

2. Put 45 c.c. of *freshly boiled* and cooled distilled water and 5 c.c. of medium in the casserole. If agar is being titrated, have the water at about 40° C. to keep the medium fluid.

3. Add 1 c.c. of indicator solution. As a general rule, there is no color change, indicating that the medium is acid.

4. Place N/20 sodium hydroxide solution in the buret and record the reading or level.

5. Add small amounts of the sodium hydroxide solution to the medium, stirring briskly after each addition.

6. Stop at the *first faint pink tinge*.

7. Read the buret and record.

8. Subtract this reading from the first to give the amount of sodium hydroxide required for 5 c.c. of medium. Example: 2.1 c.c. N/20 sodium hydroxide used. The medium has an acid reaction recorded as +2.1 according to the Fuller method.

9. If it is desired to render the bulk of the medium neutral to phenolphthalein, proceed as per the following example:

Fig. 202.—Outfit for Phenolphthalein Titration

(From Park, Williams and Krumwiede, *Pathogenic Microörganisms*, Lea and Febiger.)

5 c.c. require 2.1 c.c. of N/20 sodium hydroxide

100 c.c. require 42.0 c.c. of N/20 or 2.1 c.c. of N/1 sodium hydroxide

1000 c.c. require 21 c.c. of N/1 sodium hydroxide

10. A shorter method of calculation is as follows.

Let A equal the amount of N/20 required for 5 c.c. of medium and B the bulk of the medium:

$$\frac{A \times B}{100} = \text{c.c. of N/1 sodium hydroxide required for rendering the bulk of medium neutral to phenolphthalein}$$

11. If it is desired to have the medium slightly acid to phenolphthalein, for example, + 0.1, add 20 c.c. of N/1 sodium hydrogen instead of 21 c.c.; if an end-point of + 0.2 is desired, add 19 c.c. of N/1, etc.

12. This method, however, is only approximately correct because the buffer substances present in the medium combine with some of the sodium hydroxide so that one does not know exactly how much acid has been neutralized nor the actual

acidity of the medium. For this reason the hydrogen ion method of titration is to be preferred.

13. Few media are naturally alkaline to phenolphthalein. that is, yield a red color upon the addition of this indicator. But in this event titrate with N/20 solution of hydrochloric acid to determine the quantity required for reducing to neutrality (faint pink color), and calculate as above. If, however, a medium has been rendered too alkaline by the addition of too much sodium hydroxide, it is better to discard it than to reduce the alkalinity with hydrochloric acid, as this generally renders the medium unsatisfactory for the cultivation of sensitive bacteria.

14. The above is the *room temperature* or *standard method* to be applied to the titration of culture media brought to the boiling point in preparation before titration. If, however. the medium has been heated to only about 50° C. for dissolving the ingredients, it is necessary to boil the mixture of 5 c.c. in 45 c.c. of distilled water for one minute before the titration is conducted (the *boiling method*) to allow for the chemical changes to which the bulk of medium will be subjected during sterilization.

Methods of Determining Reaction of Bacterial Cultures.—To determine the reaction of bacterial cultures a special technic is not necessarily required. Certain factors, however, make it convenient and often advisable to use a special technic. This is especially true where the volume of the culture is small and repeated determinations have to be made. In such cases the sample for each test is very small and must be taken with aseptic precaution. In dealing with cultures of pathogenic bacteria the tubes must be sterilized upon completion of the test and this is often inconvenient with the usual technic.

The simplest technic for determining the reaction of bacterial cultures seems to be that of Brown. A large platinum loop or Pasteur pipet is used to transfer a small quantity of the culture to a little glass cup filled with distilled water plus a drop of the desired indicator. Similar cups are filled with buffer solutions at various pHs and a drop of indicator added to each. The pH of the culture is determined by comparing the color in the cup with medium to the colors in the cups with buffers. By placing the cups on a plate of milk glass very accurate pH determinations can be made. The indicators are best made up in 60% alcohol solution. The indicator solutions should be titrated to the midpoint of their color range. The complete apparatus with directions is made by the La Motte Chemical Co. and may be bought from laboratory supply houses.

Methods of Using Indicators in Culture Media for Showing Changes in Reaction Due to Bacteria.—To determine the reaction of bacterial cultures. especially in carbohydrate media for fermentation studies, indicators may be added to the media before sterilization. These indicators must not be toxic and should be relatively stable. Indicators can generally not be added to anaerobic culture media because they are reduced:

(*a*) For media with a pH from 5.2 to 6.8 the best indicator is bromcresol purple, or chlorphenol red. The latter indicator is to be preferred. A concentration

of the indicator of .001% seems most satisfactory (1 c.c. of a 1% alcoholic solution per liter).

(b) For media with a pH from 6.6 to 7.6 the best indicator seems to be brom-thymol blue. The best concentration is 0.001% (1 c.c. of a 1% alcoholic solution per liter).

(c) For media with a pH from 7.1 to 8.4 the best indicator is phenol red in concentration of 0.002% (2 c.c. of a 1% alcoholic solution per liter).

CLEARING AND FILTRATION OF CULTURE MEDIA

1. Culture media should be clear, but too fine filtration may remove growth-stimulating substances.

2. Large particles may be removed by sedimentation as in the preparation of "hormone" or "vitamin" media: After heating, allow broth media to stand overnight in the icebox and decant or pipet off the supernatant fluid next day without disturbing the sediment. Allow agar to stand overnight in a *straight side* container; turn out, trim off and discard the sediment. The agar may also be allowed to sediment in a pot and may be removed with a spoon. The sediment on the bottom layer is trimmed off with a knife. The finer particles remain in suspension and no further attempt is made at clearing except filtration through a fine wire mesh.

3. Clearing is also accomplished by coagulation of albumins during heating (boiling, Arnold sterilizer or autoclave) with enmeshment of particles, or by adding an egg to each liter of medium to furnish coagulable albumin. Mix one egg in a pan with an equal amount of water and add. If dried egg albumin is used, dissolve 10 grams in 20 c.c. of water and add. The medium is then heated in Arnold sterilizer for forty-five minutes or autoclaved for thirty minutes; ordinary boiling is not as good. Clearing with egg, however, should be avoided whenever possible because of the possibility of adding sulphur and fermentable substances tending to interfere with the growth of some organisms.

Fig. 203.—FLUTED GLASS FUNNEL FOR RAPID FILTRATION

4. For the filtration of broths and similar fluid media various grades of fine and coarse filter paper may be employed with a fluted funnel (Fig. 203) or a plain funnel with a wire rack inside.

5. Agar and gelatin media must be filtered while hot and fluid. Place a small square of coarse wire netting in the funnel and cover the netting with a thin layer of absorbent cotton. Some pour boiling water through the cotton, but this is perhaps unnecessary and the water dilutes the medium. The agar is best poured through the dry cotton and filtered into flasks. By using preparations of agar which give clear solutions in water filtration of agar media can be sometimes omitted.

STERILIZATION OF CULTURE MEDIA WITH HEAT

1. Culture media may be sterilized by (a) heat (autoclave, Arnold sterilizer or water bath) or (b) filtration. Volatile disinfectants like chloroform may be used

for the preservation of serum, ascites fluid, etc., but chemical disinfection has not as yet been perfected.

2. The minimum of heat should be used, as overheating may destroy growth-stimulating substances as well as caramelizing and hydrolizing sugars.

3. *Autoclaves* should be equipped with thermometers, as the temperature is more reliable than pounds of pressure. In general terms the equivalents are as follows:

$$5 \text{ pounds pressure} \ldots \ldots \ldots \ldots 107.7° \text{ C. } (226° \text{ F.})$$
$$10 \text{ pounds pressure} \ldots \ldots \ldots \ldots 115.5° \text{ C. } (240° \text{ F.})$$
$$15 \text{ pounds pressure} \ldots \ldots \ldots \ldots 121.6° \text{ C. } (250° \text{ F.})$$
$$20 \text{ pounds pressure} \ldots \ldots \ldots \ldots 126.6° \text{ C. } (260° \text{ F.})$$
$$25 \text{ pounds pressure} \ldots \ldots \ldots \ldots 130.5° \text{ C. } (267° \text{ F.})$$
$$30 \text{ pounds pressure} \ldots \ldots \ldots \ldots 133.5° \text{ C. } (274° \text{ F.})$$

4. Two kinds of autoclaves are available: (*a*) the upright for small laboratories (Fig. 204) and (*b*) the horizontal (Fig. 205) for larger laboratories. The former
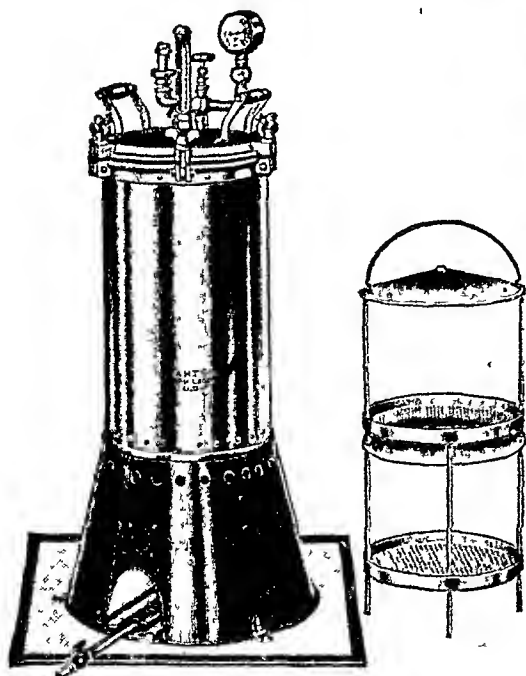
Fig. 204.—Vertical Autoclave   Fig. 205.—Horizontal Autoclave
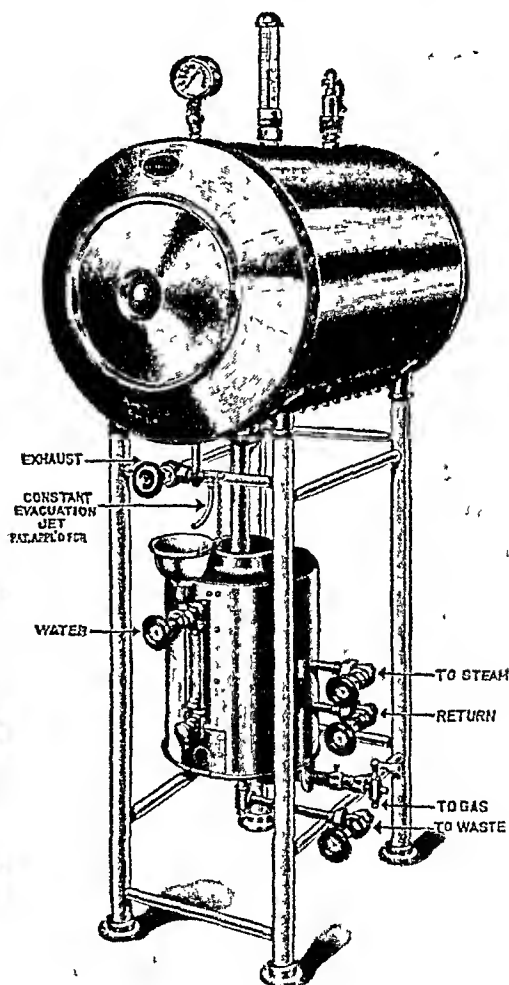
is heated with gas, kerosene, electricity (special connection required) or flowing steam; the latter may be heated with gas or connected with a steam plant. Those illustrated are recommended and full directions for operating are furnished by the manufacturers.
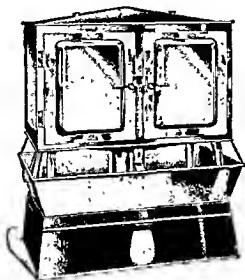
FIG. 206.—ARNOLD STERILIZER OF DOUBLE DOOR TYPE

5. For media in test tubes, sterilization at approximately 121° C. for 15 to 20 minutes is sufficient; for media in bulk, thirty minutes are required.

6. With either autoclave allow time for agar to melt before timing the period of
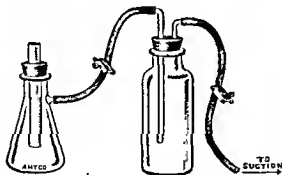
FIG. 207.—A SMALL FILTRATION APPARATUS

FIG. 208.—A WATER SUCTION PUMP

sterilization. Allow autoclave to cool before opening the door, as a sudden release of pressure may wet or blow the stoppers and crack glassware.

7. The *Arnold sterilizer* (Fig. 206) furnishes streaming steam at 100° C. and is especially recommended for routine use, as overheating is readily avoided and

likewise caramelization of sugars. The Arnold has the further advantage that the volume of liquid remains constant.

Make sure that there is plenty of water in the pan to avoid the possibility of boiling dry followed by the melting of soldered joints with danger of fire.

Media in test tubes should be heated for twenty minutes after steam is produced, and bulk media for 45-60 minutes on each of three days in succession (*fractional sterilization*), allowing extra time for the melting of agar. Allow the sterilizer to cool before removing the contents.
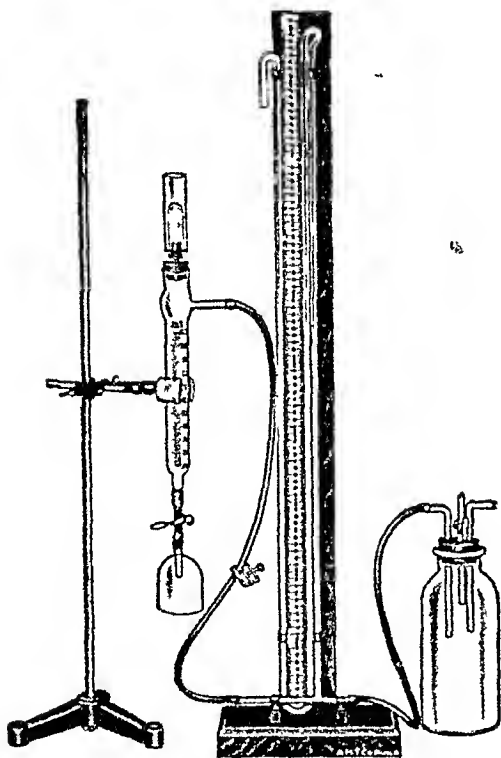
FIG. 209.—MUDD FILTRATION APPARATUS
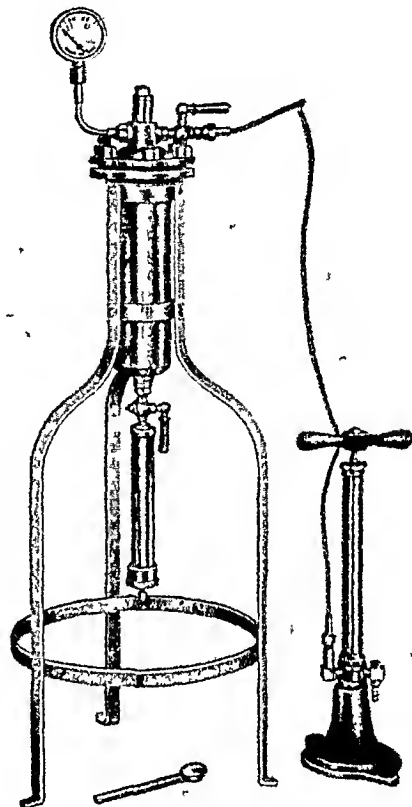
FIG. 210.—CHAMBERLAND-PASTEUR FILTER

8. *Water bath* sterilization is adapted only for small amounts of serum, ascites fluid, tissue extracts, etc., to prevent coagulation. The material should be as free as possible from contamination. This method is valueless for killing spores. The water should be above the level of the fluid to be sterilized. The temperatures should be 60° C. for about two hours.

STERILIZATION BY FILTRATION

1. Filtration methods are mainly employed for the removal of bacteria from serum, ascites and hydrocele fluids, tissue extracts, etc., that cannot be sterilized by heating at 60° C. (as when spores are present), for separating the cellular from the soluble products of bacterial growth and for sterilizing solutions of carbohydrates.

2. The filters are usually made of siliceous materials of negative electrical charge and with pores small enough to hold back bacteria and spores (Berkefeld "W"= very fine; "N" == normal or medium, and "V" == coarse for rapid filtration).

3. Suction or pressure must be provided. For small filters requiring the filtration of but small amounts of fluid, the apparatus shown in Figure 207 is usually sufficient, suction being provided by a suction pump attached to a faucet (Fig. 208).

4. For the filtration of small amounts permitting a control on pressure and volume with the sampling of filtrate at any stage, the Mudd apparatus (Fig. 209) may be recommended.

5. For the filtration of large amounts, the Chamberland (Fig. 210), Mandler (Fig. 211) and Seitz (Fig. 212) filters may be recommended. The larger sizes of these require suction or pressure pumps. The Haen is a new form employing a membrane.

6. The filter candle and all attachments with which the filtrate will come in contact must be sterile. The glassware may be sterilized in a hot air oven; the candle and rubber connections may be boiled for an hour or autoclaved.

7. New candles should be cleansed before use by passing through distilled water, followed by placing in cold water and boiling for 30 to 60 minutes.

Fig. 211.—Sectional View of Mandler Filter Cylinder

Fig. 212.—S e i t z - Uhlenhuth Filter

It is sometimes advisable to test for impermeability to bacteria with a broth culture of *B. prodigiosus*.

8. Paraffin, petrolatum, and other oils must be carefully avoided, as they tend to increase permeability to bacteria.

9. After use, candles should be cleansed by filtering through distilled water (to remove soluble and especially coagulable material) followed by sterilization by boiling (if infective material has been used) and a light scrubbing of the surface with a fine brush.

10. After continued use, candles become clogged (the average is approximately ten filtrations) and must be discarded. They may be heated to a glow, but this tends to produce cracks and increase their permeability.

11. Before candle filtration the material should be first filtered through a fine filter paper or paper pulp to remove large particles and reduce clogging.

12. Filters of the Mandler or Berkefeld types may be tested for gross leaks by immersing them in water and connecting them to a source of air pressure. A readily controlled source of air pressure may be obtained by slowly running

water from a tap into a large bottle provided with a rubber stopper. The inside of the bottle is connected through the stopper with a mercury manometer and with the filter to be tested. The pressure of the air in the bottle is gradually increased by the inflowing water and the manometer reading taken at which air bubbles first come through the filter. Defective filters are readily noted by this means and a good idea is obtained as to the condition of the filter. A good Berkefeld N filter should not pass bubbles much below a pressure of 15 inches of mercury.

FORMULAS AND DIRECTIONS FOR PREPARATION OF BACTERIOLOGICAL CULTURE MEDIA

Principles.—The preparation of bacteriological culture media is fundamental to all bacteriological work whether routine or research. Much of the care and skill which goes into the preparation of food for human consumption should go into the preparation of culture media for bacteria. Media rooms should not be located in a dark and dusty basement, as is often the case, but should be modeled on a modern kitchen. The media maker should be trained in the principles of physics and chemistry as well as bacteriology.

When directions for the preparation of media are carefully written, they should be followed to the letter. Only pure chemicals should be used except where specifically otherwise stated. All media should be prepared in scrupulously clean vessels preferably pyrex (or similar glass), enamel, or tinned copper Aluminum vessels should not be used because it has been found that they impart a certain toxicity to the medium. The reaction should be adjusted carefully, generally to within 0.1 unit of the pH specified. Sterilization should be carefully controlled as to time and temperature. In using the autoclave, it is especially important that all air be removed. It must be kept in mind that air is heavier than steam and that air pockets develop in the bottom of the autoclave and not in the top. It is also well to keep in mind that a loss of volume always takes place in the autoclave.

Media sterilized in bulk should be stored in closed containers, preferably glass, unless intended for immediate use. Crown capped milk bottles are very convenient and inexpensive for storing media. New bottles must always be heat-treated by pouring them full of boiling water and autoclaving before they are used for media. Tubed media are generally best stored in the refrigerator to lessen evaporation. The cotton plugs may also be removed following sterilization and replaced by sterile rubber stoppers. A better way is to push the cotton plug into the tube flush with the top and cover the end of the tube with a rubber cap previously dipped in 5% phenol solution. Paraffining the cotton plugs will also lessen evaporation. Concentrated sugar solutions can be kept from evaporating by means of a vaseline seal. By this means tubed media may be kept for many months following sterilization.

In every media room should be kept a book in which a detailed account is kept of each lot of medium prepared. Each batch of medium should be given a lot

number and a record should be kept of the make and lot number of every chemical which goes into the medium. The kind and age of the meat used should be recorded. The length of boiling, method of filtration, method of titration, amount of alkali or acid for the titration, pH before and after sterilization, method of sterilization, etc. should all be recorded. Tubed media should be labeled with a paper label on which is stamped or written the lot number of the medium. When large numbers of tubes of media are used over a short period of time, and if these media are simple and always made from the same basic ingredients, the tubes may be identified by staining the cotton plugs with variously colored dyes.

Peptone Water (Dunham Solution).—For indol test:

Composition.—Peptone .... 5.0 gm.
            Sodium chloride .... 5.0 gm.
            Distilled water .... 1000.0 c.c.
Adjust to pH 6.5-7.5.

Ingredients.—The peptone should have a high tryptophane content. Among the peptones on the market Difco's tryptone [4] and Wilson's C (medo) peptone seem to be best.

Method of Preparation.—Dissolve peptone and salt in distilled water by careful heating (always put water into the pan first and add the peptone to it rather than vice versa). Titrate to the desired pH, boil 10 minutes, adjust volume and filter through paper, if necessary. Tube and autoclave at 121° C for 15 minutes.

Nitrate Peptone Water.—For nitrite test:

Composition.—Peptone .... 10.0 gm.
            Sodium chloride .... 5.0 gm.
            Sodium nitrate .... 0.2 gm.
            Distilled water .... 1000.0 c.c.
Adjust to pH 6.5-7.5.

Ingredients.—Any peptone may generally be used. If medium is also to be used for indol test use same peptones as for Dunham's peptone water.

Preparation.—Same as peptone water.

Comments.—A nitrate agar is also used for this test. Variations in composition are also found such as addition of beef extract, increase of nitrate concentration, and decreased peptone concentration.

Hemo-Peptone Water.—For indol test with hemoglobinophils and other fastidious bacteria:

Composition.—Peptone .... 5.0 gm.
            Sodium chloride .... 5.0 gm.
            Distilled water .... 1000.0 c.c.
            Defibrinated blood .... 20.0 c.c.
Adjust to pH 7.3-7.6.

[4] A widely used and excellent medium for the indol test is a 0.1% solution of tryptone in distilled water at pH 7.0

Ingredients.—Peptone same as for peptone water. Defibrinated blood from rabbit, sheep, horse, etc.

Method of Preparation.—To peptone water add 2% defibrinated blood (2 c.c. to 100 c.c. of medium). Heat to 95° C. or just to boiling. Filter through paper. Sterilize by filtration through a Berkefeld or other bacteriological filter. Tube aseptically into sterile tubes. Incubate at 37° C. for 3 days to insure sterility.

Nitrate Hemo-Peptone Water.—Hemo-peptone water to which is added 0.02% sodium nitrate.

Koser Citrate Medium.—For differentiation between fecal and soil types of colon bacilli:

Composition.—Sodium ammonium phosphate (4H$_2$O)	1.5 gm.
Monobasic potassium phosphate (anhydrous)	1.0 gm.
Magnesium sulphate (anhydrous)	0.2 gm.
Sodium citrate (2H$_2$O)	3.0 gm.
Distilled water	1000.0 c.c.

Adjust to pH 6.7-6.9

Ingredients.—Only C.P. chemicals should be used.

Method of Preparation.—Simply dissolve ingredients, tube, and sterilize at 121° C for 30 minutes. No adjustment of reaction is necessary.

Malonate Medium (Leifson).—For the differentiation of the aerobacter and escherichia groups of colon bacilli:

Composition.—Ammonium sulphate	2.0 gm.
Dipotassium phosphate	0.6 gm.
Monopotassium phosphate	0.4 gm.
Sodium malonate	3.0 gm.
Bromthymol blue	0.02 gm.
Distilled water	1000.0 c.c.

Adjust to pH 6.9-7.1.

Ingredients.—Use pure chemicals.

Method of Preparation.—Dissolve salts in distilled water and add 0.2 c.c. of a 1% alcoholic solution of bromthymol blue to each 100 c.c. of medium. Tube and autoclave at 121° C. for 15 minutes.

Methyl Red—Voges Proskauer Medium.—For the differentiation of the aerobacter and eschericia groups of colon bacilli:

Composition.—Peptone	5.0 gm.
Dextrose	5.0 gm.
Dipotassium phosphate	5.0 gm.
Distilled water	1000.0 c.c.

Adjust to pH 6.8-7.1.

Ingredients.—It is probable that almost any peptone will do in this medium. Difco's proteose and Witte peptone have been recommended.

Method of Preparation.—Dissolve the ingredients in distilled water by careful heating. Titrate to required pH and boil for 10 minutes. Adjust volume and filter through paper, if necessary. Tube and autoclave at 121° C. for 15 minutes.

Nutrient Broth—A.P.H.A. Standard Method.—*Composition.*—

Peptone	5.0 gm.
Beef extract	3.0 gm.
Distilled water	1000.0 c.c.

Adjust to pH 6.6-7.0.

Ingredients.—Difco's Bacto peptone and beef extract, or other peptones or beef extracts giving comparable results.

Method of Preparation.—Dissolve the peptone and beef extract in distilled water by careful heating at about 65° C. Titrate to pH 6.8-7.2, and bring to boil. Adjust volume and filter, if necessary. Tube and sterilize in autoclave at 121° C. for 20 minutes.

Extract Broth.—For general cultivation of the less fastidious bacteria. As basis for carbohydrate broths, etc.:

Composition.—Peptone	10.0 gm.
Beef extract	3.0 gm.
Sodium chloride	5.0 gm.
Distilled water	1000.0 c.c.

Adjust to pH 7.0-7.5.

Ingredients.—Any peptone giving good general growth such as Difco's tryptone, Wilson's CB, Fairchild's, Parke-Davis, Armour, Pfanstiehl, etc.

Method of Preparation.—Dissolve ingredients in distilled water by careful heating. Titrate to desired pH and boil for 10 minutes. Adjust volume and filter, if necessary, through paper. Tube, and autoclave at 121° C. for 15 minutes.

Extract Carbohydrate Broths.—For fermentation studies of aerobic bacteria:

Composition.—Extract broth to which is added 0.5% or 1% of the desired carbohydrate, indicator, and indicator.

Ingredients.—The best general indicator is chlorphenol red or bromcresol purple. If it is desired to use the medium at a high pH, such as 7.2-7.5, the best indicator is phenol red. The best concentration for phenol red is .002% (2 c.c. of a 1% alcoholic solution per liter). Chlor phenol red and brom cresol purple are best used in .001% concentrations (1 c.c. of a 1% alcoholic solution per liter).

Method of Preparation.—Dissolve carbohydrate and indicator in extract broth. Tube (Durham tubes are generally used) and autoclave at 115° C. for 15 minutes. Some prefer to use the Arnold and sterilize by the intermittent method; however, there seems to be just as much hydrolysis of the carbohydrate by this method.

Infusion Broth.—For general cultivation of bacteria:

Composition.—Meat infusion .... 1000.0 c.c.
Peptone .... 10.0 gm.
Sodium chloride .... 5.0 gm.
Adjust to pH 7.2-7.6.

Ingredients.—Meat infusion is made from beef heart, or muscle of pork, veal, beef, etc.. by any approved method (the simplest of which is given in this book). The peptone should be one which gives good general growth of bacteria.

Method of Preparation.—Dissolve the peptone and salt in the meat infusion by careful heating. Titrate to desired pH, generally 7.6-7.8. Boil for 5 minutes, restore volume, and filter through paper. If the meat infusion has not previously been autoclaved it is necessary to titrate and boil twice in order to stabilize the reaction. Even with two boilings the pH will drop at least .2 pH units upon autoclaving. Tube and autoclave at 15 pounds for 15 minutes.

Fermented Infusion Carbohydrate Broth.—For fermentation studies with fastidious bacteria:

Composition.—*Fermented infusion broth* to which is added 0.5% or 1% of the desired carbohydrate and indicator, if desired. Generally, it is the practice to add no indicator.

Ingredients.—The ingredients are the same as for extract Carbohydrate Broth.

Method of Preparation.—For fermentation studies of the more fastidious bacteria (streptococci, meningococci, etc.) when the degree of acidity produced is generally not great, it is best to add the carbohydrates aseptically to the sterile fermented broth. The carbohydrates are best sterilized in 10% water solution with a vaseline seal or rubber cap to prevent evaporation upon storage. After addition of the carbohydrate the tubes of medium should be incubated at 37° C. for 2 days to insure sterility. Durham tubes are generally not used with this medium because it is used mostly for the non-gas producing bacteria.

Serum or Ascitic Broths.—General cultivation of fastidious bacteria:

Infusion or extract broth to which is added aseptically 2-5% of blood serum or ascitic fluid. Tubes should always be incubated at 37° C. for 2 days to insure sterility.

Serum or Ascitic Broths with Carbohydrates.—For fermentation studies with very fastidious bacteria:

Either extract carbohydrate broth or fermented infusion carbohydrate broth is used as a base. The serum or ascitic fluid is added aseptically in 2 to 5% concentration. When maltase is used, the serum added must be inactivated by heating at 60° C. for 1 hour to destroy the maltase which it contains.

Sodium Hippurate Broth.—For the test for hydrolysis of sodium hippurate:

Composition.—Infusion broth .... 1000.0 c.c.
Sodium hippurate .... 10.0 gm.

Method of Preparation.—Dissolve the sodium hippurate in the infusion broth. Tube and mark level of medium with a "non-run" wax pencil or other means on each tube. Autoclave at 121° C. for 15 minutes.

Huntoon's Hormone Broth (Modified).—For the cultivation of fastidious bacteria:

Composition.—(A) Fresh ground beef heart. (Do not remove fat
before grinding) .... 15 pounds
Eggs 9
Distilled water .... 10 liters

Stir the eggs in about one liter of water and then add to the meat and water. Stir occasionally while the mixture is slowly heated to 50° C.

(B) Peptone .... 150 gm.
Sodium chloride .... 37.5 gm.
Distilled water .... 5 liters
Bring to a boil and slowly add gelatin...... 150 gm.

Method of Preparation.—When A has reached 50° C., add B. Stir thoroughly and bring to a boil. Do not stir after the first mixing. Boil ten minutes. Remove about 500 c.c. of fluid and filter through paper. 50 c.c. portions are placed in 100 c.c. beakers. To each beaker is added an increasing amount of N/1 hydrochloric acid. Begin with 0.5 c.c. and increase by 0.25 c.c. to 1.5 c.c. One of these amounts of hydrochloric acid will cause the maximum precipitate. An aliquot portion of hydrochloric acid is added to the main portion of the mixture. Boil ten minutes. Strain through a colander. (*Do not filter through any organic material.*) Autoclave at 121° C for 15 minutes. Pour into 6 liter glass jars and allow sedimentation to proceed for 24 hours at room temperature. The supernatant medium is siphoned off leaving the fat and sediment behind. The pH is adjusted to 8.0 in the cold with N/1 sodium hydroxide and the volume is made up to 15 liters after the addition of the sodium hydroxide. Bring to a boil and pour into glass jars. In two or three hours the flocculent precipitate which has formed settles out. The supernatant is siphoned off into large flasks and autoclaved at 121° C. for 15 minutes. It is advisable to store these flasks for several days so that if a precipitate forms after the autoclaving, the supernatant can again be siphoned off. Repeated autoclaving will not affect this medium. If this broth is properly made it will support a luxurious growth of gonococcus or meningococcus without the addition of any growth promoting substances, such as ascitic fluid.

Glucose Brain Broth (Rosenow's Modified).—For isolation of fastidious bacteria, especially streptococci and microaerophilic bacteria:

Composition.—Meat infusion .... 1000.0 c.c.
Peptone .... 5.0 gm.
Sodium chloride .... 8.0 gm.
Glucose .... 2.0 gm.

Andrade's indicator .... 10.0 c.c.
Calf brain
Marble

Adjust to pH 7.0-7.5.

Ingredients.—Nutrient broth is often used in this medium in place of the meat infusion and peptone given above. The peptone should be any peptone giving good general growth.

Method of Preparation.—Dissolve peptone and salt in meat infusion by careful heating. Add indicator and glucose. Tube in fairly large tubes (20 by 1.5 cm.), the column of broth to be about 12 cm. deep. Add 3 pieces of calf brain about 1 cm. sq. and 2 or 3 pieces of crushed marble to each tube (dip the pieces of brain in water before tubing to prevent sticking to the tubes). Autoclave at 121° C. for 20 minutes. If the broth is to be used for blood cultures add 0.5% sodium citrate to prevent coagulation of the blood.

Kracke Blood Culture Broth.—For isolation of fastidious bacteria from blood:

Composition.—Heart muscle extract......................... 800.0 c.c.
Brain suspension .... 110.0 c.c.
Sodium citrate .... 1.0 gm.
Dextrose .... 10.0 gm.
Peptone .... 10.0 gm.
Di-sodium phosphate .... 2.0 gm.
Sodium chloride .... 4.0 gm.

Method of Preparation.—1. Mix the ingredients and heat until all are in solution.

2. Adjust to pH 7.4.

3. Place 50 c.c. in flasks and autoclave at 121° C. for 20 minutes.

4. Heart muscle extract:

(1) Ground beef heart (lean) 500 gms.

(2) Distilled 1000 c.c.

Place in refrigerator overnight. Press through four layers of cloth gauze. Heat the filtrate to the boiling point, and filter through small mesh wire gauze.

5. Brain suspension:

(1) Macerated beef brain 250 gms.

(2) Distilled water 500 c.c.

Place in refrigerator overnight. Filter through four layers of cloth gauze. Slowly heat the filtrate to the boiling point with constant stirring. Do not filter.

Cooked Meat Medium.—For cultivation of anaerobes.

Cut 500 gms. of lean beef or veal heart into small cubes and cover with distilled water. Bring to a boil and simmer over a low flame for 1 hour. Strain off and set

aside the fluid infusion. Pass the meat three times through a meat grinder then break up the particles by rubbing them between the hands.

To the fluid infusion add sufficient distilled water to make 2 liters and mix it with the meat. Add N/1 sodium hydroxide until the reaction of the supernatant fluid is pH 8.0. Weigh the medium in a tared vessel. Autoclave for 15 minutes. Restore the weight with distilled water. Readjust the reaction to pH 8.0. Boil for 10 minutes. Restore the weight with water and readjust the reaction to pH 8.0. Boil again for 10 minutes. Restore the weight and if the reaction is more acid than pH 7.5, readjust to this reaction.

Distribute into tubes, keeping the mixture well stirred so that there may be a uniform deposit of meat particles in each tube. Cover with a layer of sterile vaseline[5] and sterilize in the autoclave. Check the final reaction which should be about pH 7.1 and must not be acid. The repeated adjustment of reaction and boiling is necessary because the supernatant fluid contains little buffer whereas the meat particles slowly take up large amounts of alkali. The reaction of the mixture is difficult to stabilize.

Glucose Broth.—For fermentation study of anaerobes:

To infusion broth (unfermented) add 0.2% of glucose. Tube and cover with a layer of sterile vaseline about 7 mm. thick. Sterilize in the autoclave.

Starch Broth.—For fermentation study of anaerobes:

To infusion broth (unfermented) add 0.2% of "soluble" starch. Tube and cover with a layer of sterile vaseline. Autoclave.

Broth with Cube of Egg White.—For proteolytic study of anaerobes:

From the white of a hard-boiled egg cut cubes of about 5 mm. Place a cube into each tube of infusion broth. Cover with a layer of sterile vaseline. Sterilize in the autoclave.

Liver Infusion Medium (Cameron and Williams).—For general cultivation of anaerobes. Requires petrolatum seal and does not cause much sporulation of anaerobes:

Composition.—Liver infusion (as described below) . . . . 1000.0 c.c.
Peptone . . . . 10.0 gm.
Dipotassium phosphate . . . . 1.0 gm.

Method of Preparation.—Steam 500 gm. of ground beef liver with 1000 c.c. of tap water for 2-3 hours. Cool and strain through cheese cloth. Make filtrate to original volume and add 1% peptone and 0.1% dipotassium phosphate. Sterilize filtrate in flasks in autoclave for 30 minutes at 15 pounds pressure. Dry the tissue at 55-60° C. When the medium is needed, tube the infusion prepared above over

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[5] *Vaseline* is specified because not all petroleum jellies are of the right consistency or melting point for this purpose. If bacterial spores are present in vaseline they may not be killed by autoclaving because, embedded within the vaseline, they are not subjected to steam (moist heat) and the temperature of the autoclave at 18 lbs. pressure is only about 125° C. Before use, therefore, the vaseline should be sterilized in the oven (dry heat) at 165° C. for 2 hours. In our experience the vaseline may then be pipeted onto the nonsterile medium and then autoclaved without danger of contamination.

small chunks of the dried tissue and resterilize in autoclave at 121° C. for 15 minutes.

Corn-Liver Medium (McClung and McCoy).—For cultivation and isolation of all types of anaerobic bacteria without the use of petrolatum seal. Particularly adapted for isolation and study of bacteria causing spoilage of canned food:

Composition.—Corn meal	50.0 gm.
Dried liver	20.0 gm.
Distilled water	1000.0 c.c.

Components.—Ordinary whole (yellow) corn meal is used. The dried liver is obtained by drying at 55-60° C. the liver tissue obtained from the preparation of Liver Infusion (Cameron and Williams) and grinding.

Method of Preparation.—Mix the corn meal, liver tissue and water, and steam 1 hour. This is cooled and tubed as desired. The tubed medium is sterilized in the autoclave at 121° C. for 2 hours. The pressure in the autoclave should be reduced slowly to prevent blowing of the plugs.

Milk Media.—*Method of Preparation.*—1. Use fresh unpasteurized milk. preferably certified.

2. Heat in Arnold sterilizer for 30 minutes.

3. Place in refrigerator overnight.

4. Remove the cream from the top or siphon off the milk from below the cream line.

5. Tube and heat in Arnold at 100° C. for 30 minutes on three successive days.

6. Litmus milk, or bromcresol purple milk is prepared in the same manner as plain milk except that a sufficient amount of litmus solution is added to produce a distinct blue color. Bromcresol purple is added in .001% concentration.

Blood Milk.—For cultivation of hemoglobinophils:

Composition.—Sterile skimmed milk	1000.0 c.c.
Sterile defibrinated blood	20.0 c.c.
0.85% sodium chloride solution	100.0 c.c.

Method of Preparation.—A. One liter of skimmed milk sterilized in the autoclave at 121° C. for 15 minutes.

B. 20 c.c. of sterile defibrinated rabbit blood in 100 c.c. of physiological salt solution, heated just to boiling. Mix A and B with aseptic precautions and add sufficient saturated alcoholic solution of bromcresol purple to give the desired color. Tube aseptically and incubate for 3 days to test sterility.

Selenite-F Enrichment Medium.—For isolation of typhoid and paratyphoid bacilli from feces and urine.

Composition.—Sodium acid selenite (anhydrous)	4.0 gm.
Peptone	5.0 gm.
Lactose	4.0 gm.

Sodium phosphate (anhydrous)	10.0 gm.
Distilled water	1000.0 c.c.

Adjust to pH 7.0.

Ingredients.—Most any general peptone is satisfactory.

Method of Preparation.—Determine experimentally the proportion of mono- and disodium phosphate which together with the peptone and sodium acid selenite will give a pH of 7.0. The ingredients are dissolved in distilled water by gentle heat, tubed in about 10 c.c. amounts and sterilized in Arnold for not over 30 minutes.

Bile Brilliant Green Broth.—For the detection of colon bacilli in milk and water.

Composition.—Beef bile	200.0 c.c.
Peptone	10.0 gm.
Lactose	10.0 gm.
Brilliant green (1% aq. solution)	13.3 c.c.
Distilled water sufficient to give	1000.0 c.c.

Adjust to pH 7.2-7.4.

Ingredients.—The brilliant green dye should be of highest purity. The brand of peptone is probably of minor importance and any good peptone will do. Dried beef bile is to be preferred to fresh bile because it is easier to handle and more uniform.

Method of Preparation.—Dissolve the peptone and lactose in half the required distilled water. Add 20 gm. dehydrated ox bile or 200 c.c. of fresh beef bile. Measure volume and titrate to pH 7.4. Add brilliant green (13.3 c.c. of 1% aq. solution per liter) and make up to 1000 c.c. Distribute into Durham tubes in 10 c.c. amounts and autoclave at 121° C. for 20 minutes.[6]

Veal Infusion Broth.—For production of diphtheria toxin (A modification of the New York State Department of Health Laboratory Method):

Composition.—Veal, ground	500.0 gm.
Peptone (proteose-Difco)	20.0 gm.
Sodium chloride, C.P.	5.0 gm.
Maltose, C.P.	3.0 gm.
Sodium acetate, C.P.	10.0 gm.
Tap water	1000.0 c.c.

Method of Preparation.—The veal is to be freshly killed, freed of all fat and ground. The meat is infused in tap water overnight in the refrigerator. On the following morning, the meat is steamed in the autoclave (or Arnold) at 100° C. until the meat is well cooked. Usually three hours is sufficient. The coagulated blood and the fat are removed. The material is then strained and filtered through paper. This should produce a clear amber broth, which may be either light or dark, but clear.

[6] If it is desired to test 10 c.c. of water the concentration of ingredients must be increased to 1½ times that given. Such medium is tubed in 20 c.c. amounts and 10 c.c. of water added.

Measure the broth and dilute it to its original volume. Dissolve the proteose peptone and the sodium chloride and adjust the reaction to pH 7.8 to cresol red, using normal sodium hydroxide. Heat to 80° C. for 15 minutes. (Do not overheat.) Filter through paper and dilute to original volume.

Add singly the maltose and sodium acetate in concentrated solution to the broth, warm to 80° C. and filter while hot through a sterile Seitz filter in 800 c.c. amounts into sterile toxin bottles. Incubate three days at 38° C. for sterility.

Tryptic Digest Broth.—For production of diphtheria toxin (Pope and Smith).

Method of Preparation.—The meat (horse or beef muscle) is freed from easily removed fat and minced; then 40 pounds are heated with 20 liters of water in a steam cauldron to a temperature of 80° C., the mixture being occasionally stirred. When the temperature has reached 80° C. the steam is turned off and 40 liters of cold water added. The reaction of the mixture is now adjusted to pH 8.0 by means of anhydrous sodium carbonate, and the temperature is brought to 50° C. The trypsin emulsion is added in 150 c.c. quantities at half hourly intervals, a total of 12 such additions being made. Throughout the digestion the temperature is kept at 50° C. and the reaction at pH 8.0, the mixture being frequently stirred.

At 6 hours, 600 c.c. of glacial acetic acid are added and the mixture brought to boiling point; vigorous boiling is continued for 30 minutes, after which the digest is filtered through filter bags to remove undigested material.

The medium is stored overnight in the cold room. It is then returned to the steam cauldron, heated to 30° C. and the reaction adjusted to pH 7.0 with 40% sodium hydrate. Two ounces of baker's yeast are emulsified in a small quantity of the medium and then added to the main portion. The temperature is maintained at about 30° C. for one hour after which the reaction is adjusted carefully to pH 8.0. After increasing the temperature to 60° C., 0.3% maltose is added and the medium is filtered through paper and is then ready for distribution into bottles.

Trypsin Preparation.—The trypsin preparation is based on the method of Cole and Onslow (1916): 800 gms. of pancreas (which has been cleaned and very finely minced) are added to 1000 c.c. of alcohol and 2400 c.c. of water. After 3 days extraction at room temperature, 0.1% of concentrated hydrochloric acid is added and the emulsion stored in the cold room. The whole emulsion is used for digestion: filtration on a large scale proved to be very slow and moreover the emulsion appeared to be superior to the filtrate in its digestive action.

Semisolid Media.—Media containing 0.2 to 0.3% agar are known as semisolid, and are coming into more and more general use for various cultivation purposes and studies of fermentation. They seem to be especially useful in the study of the anaerobic bacteria (Spray) and in the cultivation and fermentation studies of gonococci and meningococci.

Semisolid Medium for Fermentation Tests.—For fermentation studies with gonococci and meningococci.

Composition.—Infusion broth (beef, veal, etc.) or hormone broth 1000.0 c.c.
Agar .... 2.5 gm.

| Carbohydrate | 5.0 gm. |
| Phenol red (1% alc. sol.) | 2.0 c.c. |

Adjust to pH 7.4-7.6.

Method of Preparation.—Add agar to the infusion broth and allow to soak a few minutes. Dissolve by boiling. Adjust volume and titrate to pH 7.6-7.8. Add phenol red indicator (2 c.c. of 1% alcoholic solution per liter) and tube in 5 c.c. amounts. Sterilize in autoclave at 121° C. for 15 minutes. Before use, melt the agar in the tubes by placing in boiling water and add aseptically a sterile solution of the carbohydrates. The carbohydrates may be filtered sterile or sterilized in 10% water solution.

Semisolid Agar.—For the cultivation of meningococci. Same as Semisolid Medium for Fermentation tests without the carbohydrate or indicator, and with 2.5% sterile serum or ascitic fluid added. Addition of 5% fresh tomato juice, sterilized by filtration, has been found to enhance the growth.

Nutrient Agar (A.P.H.A. Standard Method).—For enumeration of bacteria in milk and water.

Composition.—

Peptone	5.0 gm.
Beef extract	3.0 gm.
Agar	15.0 gm.
Distilled water	1000.0 c.c.

Adjust to pH 6.6-7.0.

Ingredients.—The peptone should be Bacto or other peptone giving comparable results. The beef extract should be Difco's or other giving comparable results.

Method of Preparation.—Dissolve the ingredients in distilled water by careful heating. Boil until agar is dissolved. Restore original volume and titrate to about pH 7.0. Heat to boiling and clarify, if necessary. Distribute into tubes or flasks and sterilize at 121° C. for 20 minutes.

Extract Agar.—For general cultivation of bacteria:

Composition.—

Peptone	10.0 gm.
Beef extract	3.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Distilled water	1000.0 c.c.

Method of Preparation.—Dissolve peptone, beef extract and salt in water by careful heating. Titrate to pH 7.4-7.6 and boil for 10 minutes. Adjust volume to original amount and filter through paper. Add powdered agar and allow to soak a few minutes. Dissolve by ½ hig for a few minutes and adjust volume. Tube as desired, and autoclave at 12° C. for 15 minutes. If a poor grade of agar is used it may be necessary to filter through cotton.

Sabouraud Dextrose Agar (Modified).—For cultivation and identification of fungi:

Composition.—Peptone .... 10.0 gm.
Dextrose .... 40.0 gm.
Agar .... 15.0 gm.
Distilled water .... 1000.0 c.c.

Adjust to pH 5.6-6.0.

Ingredients.—The peptone which most resembles Sabouraud's original peptone is Fairchild's. For most purposes other peptones will serve. CP dextrose is most satisfactory and is to be recommended for the sake of uniformity.

Method of Preparation.—If the peptone and agar used give clear solutions in water the ingredients are all added to the desired amount of distilled water, allowed to soak a few minutes, and carefully heated to boiling. Boil for 2-3 minutes to dissolve agar. (If peptone and agar give turbid solutions in water they had best be dissolved separately from the dextrose and filtered through cotton. The dextrose is then added to the clear agar—peptone solution.) Adjust volume and tube as desired. Autoclave at 121° C. for 10 minutes. Due to the hydrolyzing effect of acid on agar it is best not to autoclave the medium more than once. If it is necessary to remelt the medium this should be done as gently as possible.

Sabouraud's Maltose Agar. (Fisher and Arnold).—For the cultivation and identification of fungi:

Peptone .... 10.0 gm.
Maltose (technical) .... 40.0 gm.
Sodium chloride .... 7.5 gm.
Beef extract (Difco) .... 3.5 gm.
Agar .... 20.0 gm.
Distilled water .... 1000.0 c.c.

Adjust to pH 5.4.

Cornmeal Agar.—For cultivation and identification of fungi: Heat 62.5 gm. of cornmeal in 1500 c.c. of water for one hour at 60° C. Filter through paper; adjust volume to 1500 c.c. Add 19 gm. agar and heat in Arnold sterilizer one and one-quarter hour. Filter through cotton, tube and sterilize. The pH will be correct without adjustment.

Honey Agar.—For the cultivation and identification of fungi:

Honey .... 60.0 gm.
Peptone .... 10.0 gm.
Agar .... 20.0 gm.
Distilled water .... 1000.0 c.c.
Adjust to pH 5.5.
Sterilize by the fractional method.

Infusion Agar.—For general cultivation of bacteria:

Infusion broth .... 1000.0 c.c.
Agar .... 15.0 gm.

Adjust to pH 7.2-7.6.

Use finely granular or powdered agar giving a clear solution in water. Add agar to broth and after soaking for a few minutes, heat carefully and boil for a few minutes to dissolve agar. Adjust volume and titrate to pH 7.6. If this does not give a sufficiently clear medium, or if impure agar is used, the agar may be filtered through cotton or allowed to sediment overnight as described under methods of clarification.

Glucose Agar.—This may be made from either infusion agar or extract agar by adding 1% of glucose (or less, if desired).

Glycerol Agar.—For cultivation of tubercle bacilli:
Infusion agar to which is added 5% glycerol. Titrate to pH 6.8.

Extract Gelatin.—For study of gelatin liquefaction:
To extract broth add 15% of gelatin. Heat in Arnold until gelatin is dissolved. Autoclave at 121° C. for 15 minutes.

Infusion Gelatin.—For study of gelatin liquefaction:
Prepared from infusion broth in same manner as Extract Gelatin.

Lead Acetate Agar.—For study of hydrogen sulphide production:

Peptone	20.0 gm.
Beef extract	3.0 gm.
Dextrose	1.0 gm.
Basic lead acetate	0.5 gm.
Agar	15.0 gm.
Distilled water	1000.0 c.c.
Adjusted to pH 7.0.	

The peptone used should be rich in sulphur compounds (cysteine). Wilson's B, Fairchild, Proteose, or Tryptone peptones are suitable.

Dissolve the peptone in distilled water by careful heating. Titrate to pH 7.2, boil a few minutes, and filter through paper, if necessary. To the peptone solutions add the dextrose and powdered agar. Allow agar to soak a few minutes and dissolve by boiling for 2-3 minutes. Adjust volume and add 10 c.c. of a 0.5% aq. solution of basic lead acetate to each 100 c.c. of the agar. Tube and sterilize by autoclaving at 121° C. for 15 minutes.

Russell's Double Sugar Agar.—For differentiation of intestinal bacilli:

Peptone	10.0 gm.
Beef extract	3.0 gm.
Lactose	10.0 gm.
Dextrose	1.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Phenol red (1% alc. sol.)	2.5 c.c.
Distilled water	1000.0 c.c.

To melted extract agar add the lactose and dextrose. Titrate to pH 7.6 and add .25 c.c. of a 1% alcoholic solution of phenol red to each 100 c.c. of medium.

Tube so as to make a depth of medium of about 2½ inches. Autoclave at 121° C. for 15 minutes. Cool in slanting position. Andrade's indicator may be used instead of phenol red.

Krumwiede Triple Sugar Agar.—For differentiation of intestinal bacilli: Prepared as Russell's Double Sugar Agar with the addition of 1% sucrose.

Peptone Iron Agar.—For study of hydrogen sulphide production:

Peptone	20.0 gm.
Ferric ammonium citrate	0.5 gm.
Dipotassium phosphate (anhydrous)	1.0 gm.
Agar	15.0 gm.
Distilled water	1000.0 c.c.
Adjust to pH 6.8-7.2.	

The peptone used should be the same as those recommended for lead acetate agar.

The peptone, ferric ammonium citrate and dipotassium phosphate are dissolved in distilled water by careful heating. Titrate to pH 7.4, boil a few minutes and filter through paper, if necessary. Add powdered agar and after soaking a few minutes dissolve by boiling for 2-3 minutes. Adjust volume and dispense in tubes or flasks as desired. Autoclave at 121° C. for 15-30 minutes—depending upon the volume of the medium.

Simmon's Citrate Agar.—For differentiation among colon bacilli:

Magnesium sulphate (anhydrous)	0.2 gm.
Monobasic ammonium phosphate	1.0 gm.
Dipotassium phosphate	1.0 gm.
Sodium citrate ($2H_2O$)	2.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Bromthymol blue (1% alc. sol.)	2.0 c.c.
Distilled water	1000.0 c.c.
Adjust to pH 6.9-7.1.	

Dissolve the salts in distilled water and add powdered agar. Allow to soak a few minutes and then boil 2-3 minutes to dissolve. Titrate to pH 7.2. Add 0.2 c.c. of a 1% alcoholic solution of bromthymol blue per 100 c.c. of medium. Adjust volume and tube as desired. Autoclave at 121° C. for 15 minutes.

Phenol Red Tartrate Agar.—For differentiation among the paratyphoid bacilli:

Peptone	10.0 gm.
Sodium potassium tartrate	10.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Phenol red (1% alc. sol.)	2.5 c.c.
Distilled water	1000.0 c.c.

Adjust to pH 7.6. Dissolve the salts and peptone in distilled water by careful heating. Titrate to pH 7.8. Add powdered agar and allow to soak for a few minutes. Boil for 2-3 minutes to dissolve agar. Add 0.25 c.c. of a 1% alcoholic solution of phenol red. Adjust volume, tube and autoclave at 121° C. for a period of 15 minutes.

Infusion Agar with Blood, Ascitic Fluid, Serum, etc.—For cultivation of fastidious bacteria, and differentiation among streptococci:

To melted infusion agar at 45-50° C. is added aseptically 5-10% of defibrated blood, ascitic fluid, or serum. Distribute aseptically into sterile tubes or plates. Incubate at 37° C. for 24-48 hours to test sterility.

Heated Blood Agar ("Chocolate" Agar).—For cultivation of fastidious bacteria (hemoglobinophils) and differentiation among streptococci:

To melted infusion agar at 90-95° C. is added 5-10% of sterile defibrinated blood. Keep well mixed until it cools to 60° C. and then distribute aseptically into sterile tubes or plates. Incubate at 37° C. for 24-48 hours to test sterility.

Pertussis Blood Agar.—For isolation of *H. pertussis:*

Potato infusion	500.0 c.c.
Glycerol	40.0 c.c.
Sodium chloride (0.6% solution)	1500.0 c.c.
Agar	50.0 gm.

Pass peeled "old" potatoes through the meat grinder and immediately (to prevent oxidation) cover 500 gms. of the pulp with 1000 c.c. of distilled water. Add 40 c.c. of pure glycerol. Mix and then autoclave for 30 minutes at 15 pounds pressure. Filter through gauze. To each 500 c.c. of the filtrate add 1500 c.c. of 0.6% sodium chloride solution and 50 gms. of agar. Autoclave to dissolve the agar and stir thoroughly as it comes from the autoclave. Distribute into tubes, bottles or flasks. Sterilize in the autoclave at 121° C. for 15 minutes.

For use in the isolation or cultivation of *H. pertussis* melt the agar medium and when cooled to 50° C. add approximately an equal volume of sterile (not too cold) defibrinated blood. Mix thoroughly and pour into Petri dishes or slant in test tubes.

Slants to be used for the cultivation of pure cultures should be incubated to test sterility. Plates to be used for the isolation of *H. pertussis* by having a patient cough against them should be freshly poured and under these conditions the presence of a few air-borne contaminants is not likely to be confused with the growth of *H. pertussis.*

Sodium Oleate Agar (Avery).—For the isolation of influenza bacilli:

A neutral 2% solution of sodium oleate is prepared and sterilized in the autoclave. Defibrinated human or rabbit blood is centrifuged, the serum removed, and the cells made up to original blood volume with broth. To 94 c.c. of infusion or hormone agar at pH 7.4 is added 5 c.c. of the sodium oleate solution to which has been added 1 c.c. of the blood cell suspension.

Tomato Juice Agar.—For the cultivation of *B. acidophilus*:

Tomato juice (from canned tomatoes)	400.0 c.c.
Peptonized milk (Difco)	10.0 gm.
Peptone	5.0 gm.
Agar	11.0 gm.
Distilled water	600.0 c.c.

1. The tomato juice is obtained from commercial canned tomatoes. Pour the contents of the can into a large funnel with a coarse filter paper. Use the clear yellow juice filtrate.

2. Heat the mixture of tomato juice, peptonized milk and peptone to dissolve the ingredients.

3. Adjust the reaction of this mixture to pH 6.8 before adding the agar to it. Filter through paper. The final pH after sterilization will be about 6.6.

4. Add the agar to the water and boil or autoclave to dissolve the agar.

5. Combine these two mixtures while hot.

6. Filter through cotton.

7. Dispense in tubes or flasks and sterilize in the autoclave at 121° C. pressure for 8 minutes. Remove the medium from the autoclave as soon as possible.

Liver Infusion Agar.—For cultivation and isolation of Brucella:

Liver infusion	500.0 c.c.
Peptone	10.0 gm.
Sodium chloride	5.0 gm.
Agar	20.0 gm.
Distilled water	500.0 c.c.

Adjust to pH 6.6.

A. Liver Infusion—Fresh beef liver, free from fat, is ground and mixed with 500 c.c. of water (Huddleston specifies tap water but such water being variable it may be better to use distilled water). The mixture is heated in flowing steam for 20 minutes when it is thoroughly stirred. The heating is then continued in flowing steam for 1½ hours. Filter through wire screen.

B. Add the solid ingredients to the infusion and water and heat in Arnold for 1 hour. Remove and cool to 60° C. and adjust pH to 7.0. Heat again in Arnold for one-half hour. Decant off the clear agar. Put into tubes or flasks and sterilize at 121° C. for 30 minutes. The final pH will be about 6.6.

Cystine Infusion Agar.—For cultivation of *Bact. tularensis* and the Brucella:

Infusion agar	500.0 c.c.
Glucose	5.0 gm.
Cystine	0.5 gm.
Defibrinated rabbit blood	25.0 c.c.

Adjust to pH 7.4.

Add the glucose and cystine to the melted agar and sterilize in Arnold for 15 minutes with frequent shaking to dissolve the cystine. Cool to 45° C. and add the blood with aseptic precautions. Tube and slant.

Löffler's Medium.—For isolation and cultivation of diphtheria bacilli. For study of pigment production and proteolysis:

> Serum (horse, beef or sheep)   3 parts
> Extract glucose broth   1 part

Mix glucose broth and serum gently to avoid foaming. Tube in 3 to 5 c.c. amounts. Place the tubes in a slanting position in the autoclave with the layer of tubes not more than four deep. With the autoclave tightly closed the steam is turned on and pressure allowed to reach 15 pounds, where it is held steadily for 10 minutes, letting no air or steam escape. Now open the outlet valve to let air escape and simultaneously let in steam to keep the pressure constant. When all the air has escaped close the valve and sterilize at 121° C. for 20 minutes. The medium may also be coagulated at 80-90° C. in an inspissator (Fig. 213) or Arnold, after which it is sterilized by heating to 100° C. for 20 minutes on 3 successive days.
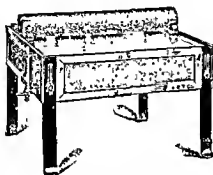
FIG. 213.—SEMI M INSPISSATOR

Tellurite Cystine Agar.—For isolation of diphtheria bacilli:

> Pork infusion agar (pH 7.4) .... 1000.0 c.c.
> Potassium tellurite (sterile 1.06% aq. sol.) .. 5.0 c.c.
> Cystine (sterile 0.25% aq. solution)........ 5.0 c.c.
> Defibrinated blood........................ 10.0 c.c.

Pork infusion agar is prepared from fresh pork muscle as described for meat infusion agar with 1.7% agar. Melt agar and add the other ingredients aseptically. Pour into plates as needed.

Tellurite Chocolate Agar (Anderson, Happold, McLeod, and Thomson). —For isolation and differentiation of diphtheria bacilli:

> Meat infusion (special) .... 1000.0 c.c.
> Peptone .... 20.0 gm.
> Sodium chloride .... 5.0 gm.
> Potassium tellurite .... 0.5 gm.
> Agar .... 20.0 gm.
> Defibrinated blood .... 100.0 c.c.

Adjust to pH 7.6.

A. Preparation of meat infusion: Add 800 gms. of fresh ground meat (kind not specified) to 1000 c.c. of water at 48° C. and keep at this temperature for 1 hour. Squeeze out juice through lint and leave juice in ice chest overnight. Filter through paper.

B. To the above infusion add the peptone and salt, and dissolve at 45° C. Adjust reaction by removing 50 c.c. sample. Heat this to 80-90° C. for 15 minutes and filter through paper. Titrate 10 c.c. of the filtrate to pH 7.6. Add required alkali to main batch. Filter through a Scitz K clarifying film, and then through a sterile Chamberland filter. Dispense aseptically into tubes or flasks and incubate same for sterility. Mix equal parts of this broth with sterile 5% agar solution. Add blood and tellurite. Heat at 75° C. for 10-15 minutes before pouring into plates.

Testicular Infusion Agar.—For isolation and cultivation of gonococci and meningococci:

Testicular infusion	500.0 c.c.
Peptone	10.0 gm.
Sodium chloride	5.0 gm.
Dextrose	5.0 gm.
Agar	20.0 gm.
Distilled water	500.0 c.c.

Adjust to pH 7.4-7.6.

Use only fresh beef testicle. Neopeptone has been found satisfactory. Other good peptones are probably satisfactory.

Add the powdered agar to 500 c.c. of distilled water. Allow to soak for a few minutes and boil until dissolved. Dissolve the peptone, salt, and dextrose in the testicular infusion by gentle heating at 50° C., preferably in a pyrex vessel. Filter through paper if necessary. Mix the two solutions and adjust the volume to 1000 c.c. Titrate to pH 7.6-7.8 (the final pH must not be below 7.4). Sterilize in tubes or bottles by steaming at 100° C. for 30 minutes on 2 consecutive days. Incubate at 37° C. for sterility.

Preparation of Testicular Infusion.—Fresh, fat-free beef testicle is weighed and ground. Add the ground testicle slowly to an equal quantity of boiling distilled water, and boil for 15 minutes. Strain and filter through paper. Standardize the broth by placing 10 c.c. in a porcelain dish with 25 c.c. of distilled water. Prepare a similar control dish. Add 5 drops of a 0.5% absolute alcoholic solution of thymolthalein to each dish. Titrate the one dish to a faint blue with N/10 NaOH. Add the calculated amount of N/1 NaOH to the bulk of the broth to bring it to the same pH (9.3). Heat at about 50° C. for 10 to 20 minutes for the phosphates to precipitate. Filter through fine paper and measure quantity. Adjust reaction to pH 7.9-8.0. Place in flasks and sterilize by steaming for 30 minutes on two consecutive days. Incubate to test sterility.

Potato Medium.—For differentiation of bacteria:

Wash and peel large potatoes. Cut out cylindrical pieces with a cork-borer.

Then cut the cylinders diagonally with a knife so as to make slants. Immediately immerse the slants in distilled water. Change the water several times and soak the slants overnight in the refrigerator. Wash the slants again in fresh distilled water. Place the slants into test tubes and sterilize in the autoclave.

(The washing and soaking of the potato in water serves to prevent darkening of the potato. A small pledget of wet absorbent cotton in the end of each tube below the potato slant will help to prevent drying during storage.)

Dorset Egg Medium (Modified).—For cultivation of tubercle bacilli:

Soak 6 fresh eggs in 1:1000 mercuric chloride solution and allow the antiseptic to drain off on a sterile towel.

Break the eggs carefully and, as nearly aseptically as possible, add the yolks and whites to 100 c.c. of sterile 5% glycerol in distilled water contained in a wide mouth flask or beaker. Break up the yolks and mix thoroughly by means of a sterile spatula and by shaking the flask but avoid foaming. Tube, slant and inspissate for about 2 hours at 70-75° C. on three successive days or slant in a horizontal autoclave, close the autoclave tight and, without allowing the air to escape, autoclave at 121° C. for 15 minutes on each of three successive days. The original Dorset medium did not contain glycerol but there are many modifications of the medium. The one described resembles that of Soparkar (*Ind. J. Med. Res.*, 1916-17, iv, 28) except that it contains less egg.

Petragnini Medium.—For isolation of tubercle bacilli:

Potato (peeled and cut into small pieces)	75.0 gm.
Milk (cream removed)	150.0 c.c.
Potato flour	6.0 gm.
Peptone	10.0 gm.

Mix and heat in a double boiler for ten minutes with frequent stirring. After mixture becomes pasty, continue to heat for one hour. Add sterile distilled water to make up volume. Cool to 50° C. To above add the following mixture:

Eggs (whole)	4
Egg yolk	1
Glycerol	12 c.c.
Malachite green (2.0% aqueous)	10 c.c.

Mix thoroughly and filter through sterile gauze into a sterile distributing funnel. Distribute into rather large test tubes. Place in a slanted position in an inspissator or Arnold and heat for two hours at from 70 to 75° C. on three successive days.

Whey Agar.—For cultivation of lactobacilli (acidophilus, etc.):

Skim milk	1000.0 c.c.
Peptone	5.0 gm.
Agar	15.0 gm.

Adjust to pH. 6.0-6.5.

Autoclave the skim milk for 3-4 hours at 15 pounds of pressure. Filter off the precipitated protein and to the clear filtrate add 0.5% peptone and 1.5% agar.

Allow to soak a few minutes and heat carefully to boiling. Boil to dissolve agar, adjust volume and tube as desired. Autoclave at 121° C. for 15 minutes or longer.

Eosin Methylene Blue Agar.—For isolation of intestinal pathogens:

Peptone	10.0 gm.
Dipotassium phosphate	2.0 gm.
Lactose	5.0 gm.
Sucrose	5.0 gm.
Eosin y	0.2 gm.
Methylene blue	0.05 gm.
Agar	15.0 gm.
Water	1000.0 c.c.

Adjust to pH 6.8-7.7.

Dissolve the peptone and phosphate in distilled water by careful heating. Titrate to pH 7.2, boil a few minutes and filter, if necessary. Add the agar, allow to soak a few minutes and boil until dissolved. Adjust volume. Add the lactose and sucrose. To each 100 c.c. of medium add 1 c.c. of a 2% solution of eosin y and 1 c.c. of a 0.5% solution of methylene blue. Tube in 15 c.c. amounts or put into flasks and sterilize in the autoclave.

Desoxycholate Agar.—For isolation of intestinal pathogens:

Peptone	10.0 gm.
Agar	16.0 gm.
Sodium desoxycholate	1.0 gm.
Sodium chloride	5.0 gm.
Dipotassium phosphate	2.0 gm.
Lactose	10.0 gm.
Ferric Ammonium citrate	2.0 gm.
Neutral red (certified) 1% alc. sol.	3.3 c.c.
Distilled water	1000.0 c.c.

Adjust to pH 7.2-7.4.

Proteose, Wilson's CB, and Fairchild's peptones are satisfactory.

Dissolve the peptone in distilled water by gentle heating. Titrate to pH 7.5, boil and filter, if necessary. Dissolve agar (use powdered agar) in the peptone solution and dissolve by boiling. Add the other ingredients in the order given and dissolve. Adjust volume and titrate to pH 7.5. Add neutral red (3.3 c.c. of 1% aq. solution per 1000 c.c. of medium). Distribute in tubes or flasks and Arnolize for 15 minutes. Spore formers will not grow in this medium and the sterilization need only kill vegetative cells.

Desoxycholate Citrate Agar.—For isolation of intestinal pathogens:

Pork infusion (special)	1000.0 c.c.
Peptone	10.0 gm.
Sodium desoxycholate	5.0 gm.
Sodium citrate (2H₂O)	20.0 gm.
Lactose	10.0 gm.

Ferric ammonium citrate	2.0 gm.
Neutral red (certified) 1% aq. sol.	2.0 c.c.
Distilled water	1000.0 c.c.

Adjust to pH 7.3-7.5.

The best peptone to use is Wilson's B peptone because with this peptone colonies of typhoid bacilli are characteristically translucent while with other peptones they are opaque. Proteose is good but gives opaque typhoid colonies.

To the specially prepared pork infusion add 1% peptone and dissolve by gentle heating. Titrate to pH 7.5, boil and filter, if necessary. Add powdered agar and dissolve by boiling. Dissolve the sodium desoxycholate in distilled water to make about a 20% solution. Add this solution to the melted agar and then the other ingredients, except the neutral red, in the order given. Adjust volume and titrate to pH 7.3-7.5. Add neutral red (2 c.c. of a 1% aq. solution per liter). Tube in 15 c.c. amounts and sterilize in Arnold for 15 minutes, or, preferably, heat to boiling and pour directly into plates. Store in refrigerator in the dark. Heat is detrimental to medium and the less it is heated the better.

Preparation of Pork Infusion.—To fresh, lean, ground pork is added 3 times its weight of water and ½ c.c. of N/1 HCl per 100 grams of meat. Mix meat and water and allow to infuse for about 1 hour and then boil for 5 minutes. Strain, and filter the cooled infusion through paper until clear and free of visible fat. Add alkali to bring pH to 8.0, boil 10 minutes and filter through paper. Restore volume to that of water originally added and titrate to pH 7.4. Tube in measured amounts and autoclave. Any precipitate which forms should be filtered off before use.

Desoxycholate Lactose Agar.—For direct enumeration of colon bacilli in milk and water:

Peptone	10.0 gm.
Agar	15.0 gm.
Lactose	10.0 gm.
Sodium desoxycholate	0.5 gm.
Sodium chloride	5.0 gm.
Sodium citrate	2.0 gm.
Neutral red (certified) 1% aq. sol.	3.5 c.c.
Distilled water	1000.0 c.c.

Use a good general peptone such as Wilson CB, Proteose, Fairchild, etc.

Dissolve peptone in distilled water by careful heating and titrate to pH 7.3-7.5. Boil and filter, if necessary. Add powdered agar and dissolve by boiling. Dissolve other ingredients in the melted agar, adjust volume and check pH (7.3-7.5). Add neutral red (3.5 c.c. of a 1% aq. solution). Dispense in tubes or flasks as desired and autoclave at 121° C. for 15-30 minutes depending upon volume.

Triple N.N.N. Medium.—For the cultivation of the Leishmania (Novy, Mac-Neal and Nicolle):

Agar	14.0 gm.
Sodium chloride	6.0 gm.
Distilled water	900.0 c.c

After tubing and sterilizing in the autoclave, the medium is cooled to about 50° C., and one third volume of sterile whole rabbit blood is added. After mixing well the tubes are set in slants. No adjustment of pH.

Locke-Egg Serum Medium.—For the cultivation of protozoa:

1. Take four eggs, wash with tap water, brush with alcohol, and carefully break into a sterile flask containing glass beads.

2. Add 50 c.c. of Locke's solution.

3. Shake the mixture until the egg is thoroughly broken up.

4. Pour into test tubes a sufficient quantity to produce a slant 1 to 1½ inches in length.

5. Slant the tubes in an inspissator, heat at 70° C. until the mixture is solidified.

6. After complete coagulation, autoclave at 121° C. for 20 minutes, or by fractional sterilization in the Arnold. Care must be taken to raise the temperature in the autoclave very slowly. Care likewise must be exercised to allow the autoclave to cool slowly.

7. Cover the media in the tubes with a mixture prepared as follows: Take equal parts of sterile Locke's solution and sterile inactivated blood serum. Mix and pass through a Berkefeld filter. Incubate the mixture to determine sterility. If sterile, the mixture is ready to be added to the tubes.

Formula of Locke's Solution.—

Sodium chloride	9.0 gm.
Calcium chloride	0.2 gm.
Potassium chloride	0.4 gm.
Sodium bicarbonate	0.2 gm.
Glucose	2.5 gm.
Distilled water	1000.0 c.c.

Solution is filtered and autoclaved at 121° C. for 15 minutes, and allowed to cool before use.

Long's Synthetic Medium.—For the cultivation of tubercle bacilli.

Asparagin	5.0 gm.
Ammonium citrate	5.0 gm.
Potassium acid phosphate	3.0 gm.
Sodium carbonate (anhydrous)	3.0 gm.
Sodium chloride	2.0 gm.
Magnesium sulphate	1.0 gm.
Ferric ammonium citrate	0.05 gm.
Glycerol	50.0 c.c.
Water	1000.0 c.c.

Tube and sterilize at 121° C. for 15 minutes.

BACTERIOLOGICAL SOLUTIONS AND REAGENTS

Sodium Chloride (Physiological Salt) Solution.—

Sodium chloride (C.P.) . . . .   8.5 gm.
Distilled water . . . .   1000.0 c.c.
Arnold or autoclave sterilization.

Buffer Solution.—

Sodium dihydrogen phosphate (NaH$_2$PO$_4$) . .   26.81 gm.
Disodium hydrogen phosphate (Na$_2$HPO$_4$) . . .   125.0 gm.
Distilled water . . . .   1000.0 c.c.
Arnold sterilization

Sodium Chloride Solution (Buffered).—

Buffer solution (above) . . . .   20.0 c.c.
Sodium chloride (C.P.) . . . .   8.5 gm.
Distilled water . . . .   1000.0 c.c.
Arnold or autoclave sterilization.

Sodium Citrate-Sodium Chloride Solutions.—

	1%	2%	3%
Sodium citrate (C.P)	10 gm.	20 gm.	100 gm.
Sodium chloride (C.P.)	8.5 gm.	8.5 gm.	8.5 gm.
Distilled water	1000 c.c.	1000 c.c.	1000 c.c.

Arnold sterilization.

Potassium Oxalate Solution.—

Potassium oxalate . . . .   2.0 gm.
Sodium chloride . . . .   6.0 gm.
Distilled water . . . .   100.0 c.c.
Arnold sterilization.
1 c.c. of this solution prevents coagulation of 10 c.c. of blood.

PREPARATION OF PERCENTAGE SOLUTIONS BY DILUTION

1. From the concentrated solution of known strength, take the number of cubic centimeters corresponding to the percentage desired in the new solution.

2. To this, add distilled water until the total number of cubic centimeters equals the percentage numeral of the concentrated solution. For example, to prepare a 0.5 per cent solution from a 10 per cent solution, take 0.5 c.c. of the latter and add 9.5 c.c. of distilled water.

Preparation of Percentage Solutions of Alcohol.—1. To prepare from 96% alcohol:

$$X = \text{desired amount of diluted alcohol}$$
$$Y = \text{desired percentage (expressed as a decimal)}$$
$$\frac{X \times Y}{.96} = \text{amount of 96 per cent alcohol required}$$

7. In preparing smears of cultures, place a loopful of water on a slide; with a sterile wire transfer a small amount of culture to give an opalescent suspension; spread into a thin layer. The water should be essentially sterile. preferably distilled and not more than 3 days old.

8. Allow smears to dry in the air or with the aid of gentle heating. A slide may be dried by holding it *with the fingers* above a Bunsen flame, since a degree of heat bearable by the fingers will not "cook" or harm the smear (Fig. 216).
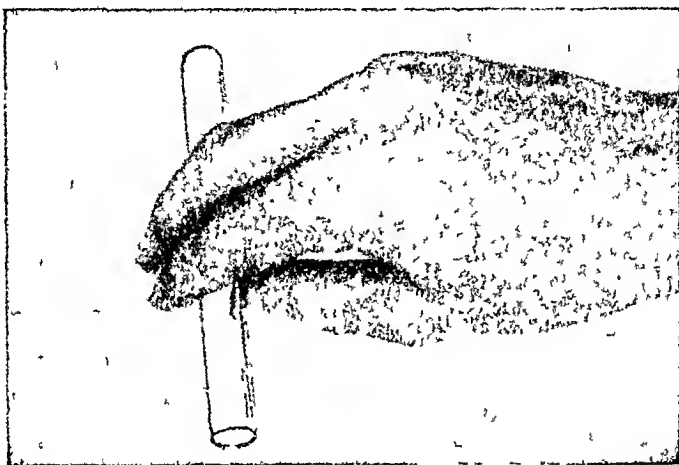
Fig. 215.—Thoroughly Draining Sediment in Bottom of Centrifuge Tube before Preparing Smears

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

9. Do not use the filthy and dangerous method of covering a thick wet smear with another slide.

10. Stained smears keep indefinitely. but if mounted in Canada balsam, cedar oil, or dammar lac they tend to fade unless the preservative is *neutral*.

METHODS OF CULTIVATING AND ISOLATING BACTERIA AEROBICALLY

1. In transferring bacteria from a culture to a tube or plate of medium, certain precautions must be taken to prevent contamination by outside bacteria. Laboratory air always contains a considerable number of bacteria, and the dust on tables or chairs is full of bacteria. To minimize the chance of contamination. it is advisable to keep tables. chairs and window sills freshly washed. Special "sterile" rooms are often used in which to transfer bacteria. It is also well to keep in mind that when pathogenic bacteria are handled these must not be allowed to escape from the culture and be scattered over the table and into the room. Work as quietly as possible and observe at all times certain precautions and technic.

2. Pasteur transferred bacteria from a culture to a fresh medium by means of a pipet, now known as a "Pasteur pipet." Since the time of Koch the wire needle or loop has been more generally used. These needles are made of platinum or nickel-chromium steel (nichrome, or stainless steel) about 0.025 inch in thickness. The

CHAPTER XVIII

GENERAL BACTERIOLOGICAL METHODS

Principles.—1. While general bacteriology is an exceedingly complicated subject by reason of the very large number of bacteria known to exist, the number of bacteria producing disease in human beings and the lower animals is comparatively small. The majority of these bacteria are readily detected and identified by present-day methods.

2. Many may be identified by the proper staining of the exudates they produce, supplemented by a study of their cultural and biological characteristics. The technic is relatively simple but demands the employment of accurate methods, including proper methods of staining and differential staining.

3. A good microscope equipped with a satisfactory oil-immersion objective and proper illumination are essential; it is a mistake to temporize with poor objectives, eyepieces and inadequate lighting.

PREPARATION OF SMEARS OF EXUDATES

1. The examination of stained smears of pus, sputum and other exudates is usually of value in bacteriological examinations and diagnosis; in some instances it constitutes the chief means of diagnosis as in gonorrhea, Vincent's angina, spirofusillar gingivitis, acute contagious conjunctivitis, tuberculosis, leprosy, etc.

2. Slides are preferred to coverglasses as they are less breakable, more easily handled and readily filed. They should be clean and not too badly scratched. Used slides may be used after cleaning as described in Chapter XVI.
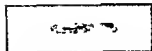
FIG. 214.—SMEAR WITH THIN AND THICK AREAS.

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis,* Waverly Press, Inc., Baltimore.)

3. Smears may be prepared with sterile cotton swabs or with flamed stiff wire loops. At least two should be prepared on the same or separate slides.

4. It is important to have smears neither too thick nor too thin. They need not be larger than 1 cm. in diameter if the material is scanty (Fig. 214).

5. *Vigorous rubbing should be avoided* as the cells may be broken up and intracellular examinations made difficult or impossible. This is especially important in examinations for gonococci and meningococci or when making a differential count of cells for cytodiagnosis. *The swab should be rolled on the slide* and should not cover the same area twice.

6. Cerebrospinal fluid and other transudates and exudates poor in cells may be first centrifuged and smears prepared of the sediment (Fig. 215).

4. If gelatin is being used and if it is somewhat dry, it may be first melted in hot water and allowed to resolidify; inoculate with a deep puncture.

5. If the medium is semisolid. make a deep puncture with a loop, or if a pipet is used, expel the material slowly as the pipet is withdrawn.

6. If the medium is liquid. suspend the material in it with a loop or pipet.

7. When a large amount of fluid material is to be transferred. use a sterile pipet with a cotton plug. When the material is very infectious, attach a piece of rubber tubing with mouthpiece to the pipet or use a Pasteur pipet fitted with a
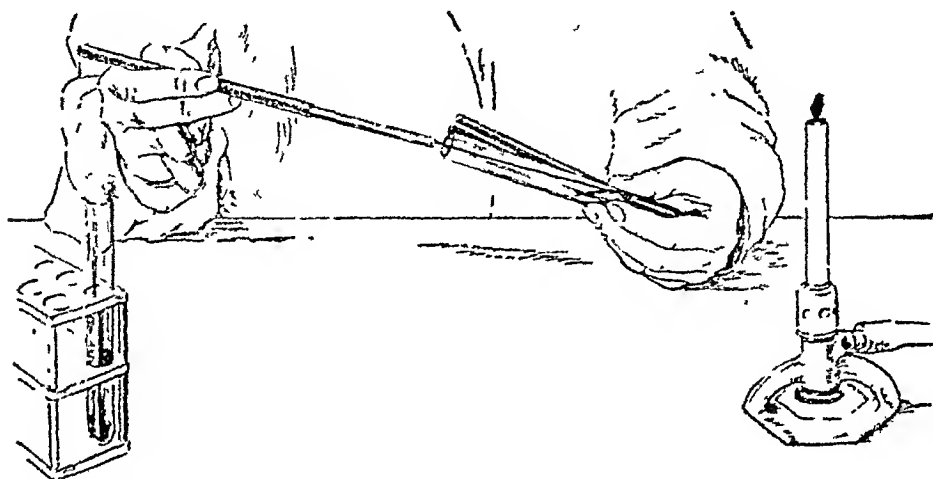
Fig. 219.—Method of Holding Tubes and Wire for Inoculation of Tubes
(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

rubber bulb. As soon as the culture has been made, place the pipet in a jar containing a disinfectant. If material is accidentally taken into the mouth, rinse thoroughly with water, then 5% phenol, 40 to 50% alcohol. and again with water.

8. If a Petri plate is to be inoculated, raise the cover at one side just high enough to admit the wire or swab, keeping the plate as completely covered as possible to prevent contamination from the air (Fig. 220).

Surface Streak Plates.—Streak plates are made for the purpose of studying colony formations of bacteria and to isolate different bacteria from a mixture.

1. The first essential for making a good plate is to get the proper dilution of the bacteria. Dilutions may be made either in sterile distilled water or in sterile broth. For delicate bacteria the latter is preferred. For making dilutions of various kinds of exudates one may have to rely much on guess work. By examining stained smears of the exudate under the microscope a fair idea can be obtained of the number of bacteria it contains and dilution can be made accordingly. It is, however, simpler to streak a number of plates in series.

2. The streak is best made by the wire loop bent at the tip to form a flat surface about 1 cm. long. The inoculation is made by making eight or ten streaks over the surface of the plate. The loop should be allowed to rest lightly when

latter is better and also less expensive. The wire needles are sterilized in the flame by heating to red heat. The lower part of the handle should also be sterilized. The Kolle (Fig. 217) and Rosenberger and Greenman (Fig. 218) holders are recommended (glass handles are very unsatisfactory because of cracking).

Fic. 216.—Touching a Heated Slide to the Back of the Hand to Judge the Temperature and Avoid "Cooking"

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

The tubes should be held almost parallel with the table top to avoid air contamination (Fig. 219).

Remove the plugs (do not flame) and hold them between the third and fourth fingers of the right hand; now flame the ends of both tubes (but not too long, as cracking may occur); transfer the material; reflame the ends of the tubes and replace the stoppers. When making smears replace the plugs before spreading the

Fic. 217.—Kolle Wire Holder

Fic. 218.—Rosenberger and Greenman Wire Holder

material on the slide. It is not necessary to flame the stoppers before replacing them. If they are flamed, however, be sure to hold the test tube end of the plug low down in the flame to prevent the loose cotton held by the fingers from catching fire. Be sure that the plugs are inserted so deeply that they will not become loosened. Label properly and preferably with gummed labels as pencil markings may be rubbed off.

3. Be careful not to break the surface of a solid medium when inoculating slants. (The butt, however, may be punctured.)

the diluted material is pipetted directly into the sterile Petri dish and the melted agar at 45° C. is poured on top and mixed (Fig. 221). Generally 10 to 12 c.c. of agar is used in each 15 mm. dish.

3. For studying colony formations, as with *blood agar*, the procedure is as follows: to the melted agar at 45-50° C. is added 5 to 10% of defibrinated blood (citrated blood is also used). The bacteria, properly diluted, are then added to the blood agar and mixed by rolling the tube between the palms of the hands or by swinging the tube with a circular motion, taking care to prevent foaming of the
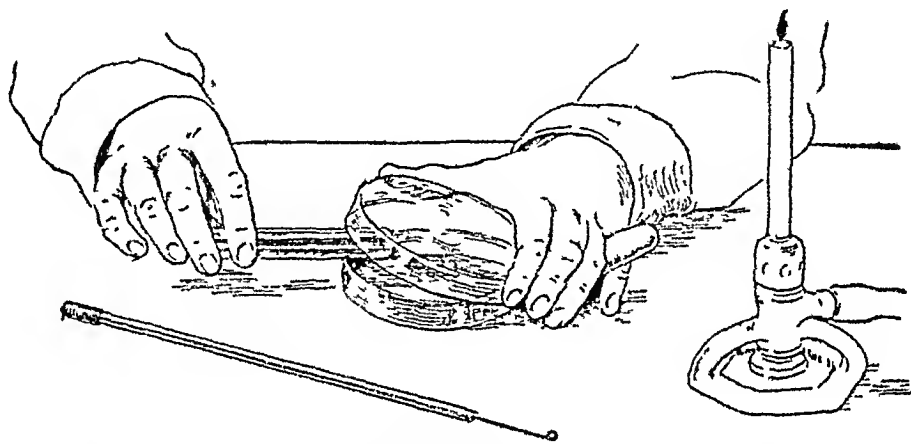
Fig. 221.—Method of Pouring Inoculated Medium into a Sterile Petri Plate

(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

medium. The inoculated blood agar is then poured into the plate, taking care to flame the mouth of the tube. The drop of agar which generally is left on the mouth of the tube should not be allowed to run down the outside of the tube as it generally will, but should be wiped off with the bottom of the cotton plug. Proper dilutions are usually obtained by putting a loop of the culture into 5 to 10 c.c. of sterile water or broth and taking a loop of this into the agar.

4. Or, the following method may be used: Into one of the tubes of melted and cooled agar place a loopful of the material to be examined. Mix well by rotating between the palms of the hands or by very careful shaking to insure uniform distribution. After flaming the wire, transfer three loopfuls from this tube to a second and mix thoroughly; repeat by placing five loopfuls into the third tube from the second. (This must be carried out rapidly as the agar may solidify before the transfers are completed and thus interfere with the next step.) Pour the agar from each tube into a separate Petri dish, taking care to flame the mouth of each tube and to lift the lids of the plates just enough to admit the end of the tube; distribute evenly by gently rotating and tilting. Allow the agar to solidify, and place the dishes in the incubator, cover side down. If colonies have developed sufficiently at the end of 24 hours, examine both surface and deep colonies on whichever plates

being drawn over the medium to prevent cutting the surface. Where only one plate is streaked, it is best to divide the plate into four quadrants and streak in series. If many plates are used, it is very simple to obtain the right dilution.

3 Another good method is to place one to five loopfuls of the material on the
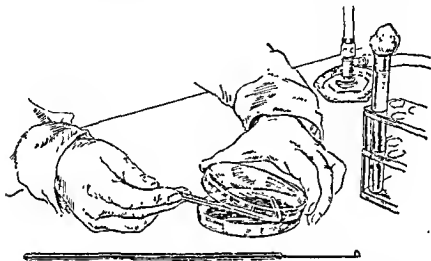
FIG. 220.—METHOD USED IN SPREADING A DROP OF INOCULUM OVER THE SURFACE OF MEDIUM IN A PETRI PLATE BY MEANS OF A STERILE GLASS ROD

(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

surface of the medium and by means of a bent glass rod, which can be sterilized by flaming, spread the drop over the plate. If many organisms are likely to be present, one or more plates may be smeared without placing any more material on the rod.

4. The surface of streak plates should be fairly dry. A dry surface is obtained by storing for a few days or by using porous porcelain covers, preferably glazed on the outside. The plates should be placed in the incubator upside down.

Pour Plates.—Pour plates are made when it is desired to study colonies of bacteria in the agar rather than on the surface, and for the enumeration of bacteria in a culture, or in milk, water, etc.

1. The agar medium in tubes or flasks is melted by immersion in boiling water for a few minutes, or in the Arnold or autoclave. It is then cooled to a temperature between 45° and 50° C. In large laboratories, or when it is desired to have melted agar ready at all times, the method proposed by Magath (*J. Lab. & Clin. M.*, 1928, 13: 672) will be found useful. Two heaters, of about one quart capacity each, are so wired with a switch and lamp that in one position of the switch water is boiled in both containers and, at another position of the switch, the temperature is reduced to about 46° C.

2. For quantitative work, as in the enumeration of bacteria in milk and water,

Liquid Tubes with Marble Seal (Hall).—Special tubes are used with a constriction below the middle. In this constriction is placed a glass ball or marble. The medium below this seal is generally quite free of oxygen.

Alkaline Pyrogallate Methods.—The oxygen may be absorbed by alkaline pyrogallol in individual agar tubes as in the so-called *Wright tube.* The agar in such tubes may be inoculated either as stabs or slants, usually the latter. The cotton plug is clipped off with a pair of scissors and pushed into the tube for a short distance. On top of the plug is then placed a small quantity of pyrogallic acid and sodium carbonate. Just before inserting a tight-fitting rubber stopper, a small quantity (1-2 c.c.) of water is poured in and the rubber stopper pushed in tight. A small quantity of sodium hydroxide may be used instead of the carbonate and water. The tube should be incubated with butt end up.

Spray Plate Method.—This consists of a modification of the McLeod method and employs a specially designed plate made of pyrex glass consisting of the bottom of a Petri dish and a special dish with an impressed ridge on the bottom and a rolled moat around the top (*J. Lab. & Clin. M.,* 1930, 17: 203).

1. These two parts are assembled, wrapped with paper and sterilized by baking.

2. Pour the inoculated blood agar or other medium into the Petri dish and immediately invert the bottom of the apparatus over it while the agar hardens. Hardened plates may be surface streaked.

3. Invert; raise the Petri dish and on one side of the ridge place 4 c.c. of 40% pyrogallic acid and on the other side 10 c.c. of 20% sodium hydroxide. Replace the Petri dish and seal by pouring around the moat, hot paraffin, a mixture of two parts paraffin and one part beeswax or a good grade of oil clay. One may seal in a string with the ends protruding from the paraffin or clay to aid in opening the dish later.

4. Tip the dish to mix the solution and incubate.

5. The plate may be examined repeatedly without opening and when proper growth has developed the plate is pried off or the string pulled to cut the seal and the colonies examined.

Anaerobic Jar Methods.—1. First heat the medium in boiling water for 15 to 20 minutes to drive off oxygen and cool to 42°-45° C. before inoculating. Incubate in a jar from which the oxygen has been removed. Any large jar with a tight fitting cover or stopper may be made into an anaerobic jar by the use of alkaline pyrogallate.

The *Novy jar* (Fig. 222) is an example of a jar in which the oxygen is displaced by hydrogen or other inert gases such as nitrogen. These jars are generally first evacuated, then filled with hydrogen, again evacuated, and finally filled with hydrogen.

The *McIntosh and Fildes jar* illustrates the type of jar in which the oxygen is removed by combination with hydrogen by means of a platinum-block catalyst (Fig. 223).

The *Weiss-Spaulding* apparatus shown in Figure 224 is satisfactory and can be

they are separated sufficiently to permit of fishing and subculturing. Most of the colonies with this method of plating will be deep and very difficult to differentiate by their growth.

Isolation of Aerobic Spore-Forming Organisms.—When material is known or suspected of containing spore-forming bacteria and is likely to be contaminated with other bacteria, a part, suitably diluted with sterile saline or broth, may be heated at 80° C. in a water bath for 30 minutes, or at 70° C. for one or two hours, to destroy the nonsporulating or vegetative forms. Then proceed by any of the above methods for isolating pure cultures.

METHODS OF CULTIVATING AND ISOLATING BACTERIA ANAEROBICALLY

The anaerobic bacteria are those which will not develop unless the oxygen concentration is reduced to a very low value. Such conditions are generally obtained by placing the inoculated tubes or plates in air-tight jars fitted with some system for removing the oxygen. *The best method for doing this is either chemically by alkaline pyrogallol, or by combining the oxygen with hydrogen using a catalyst.* Deep tubes of broth, especially if they contain particles of meat, etc., or agar freshly boiled, will generally grow most anaerobes. Such tubes of broth are generally covered by a "seal" of petrolatum or a petrolatum-paraffin mixture to decrease the rate of diffusion of oxygen into the medium.

Shake Tube Method.—Melt deep agar tubes by placing them in boiling water for several minutes. Cool to about 45° C. and inoculate as directed for making "pour" plates. Allow to harden and incubate. Sufficiently anaerobic conditions are usually found in the deeper layers of medium to cause good growth of most anaerobes. Separate colonies may readily be fished by expelling the agar into a sterile Petri dish. This is best done by means of a pipet similar to a Pasteur pipet which is made from glass tubing. The tip of the pipet should be drawn rather thin and the end sealed in the flame. Gently heat the bottom of the tube in the flame to melt the agar slightly and to loosen it. Push the tip of the pipet through the agar and hard against the bottom of the tube so that the end of the pipet is broken. Now blow through the pipet and withdraw it as the agar is pushed from the tube. By means of the pipet the agar is transferred to a sterile Petri dish. With a sterile wire the agar may be cut in any desired place to expose the colonies desired and permit fishing.

Liquid Tubes with Petrolatum Seal.—The medium is preferably freshly boiled to drive out the oxygen. It is then allowed to cool in a slanting position. Since it is impractical to inoculate the medium through the petrolatum seal it is best to make an opening in the seal. This is best done by gently warming the bottom of the slanted seal which then flops up leaving a clear opening into the medium. When inoculated the petrolatum is gently warmed and allowed to cover the medium.

alve. When combustion is complete. the hydrogen inlet is closed. the electric urrent turned off and disconnected. and the jar is ready to be incubated. When ised in this manner the atmosphere within the jar consists almost wholly of ıydrogen. If it is desired to retain an atmosphere composed of nitrogen and a'
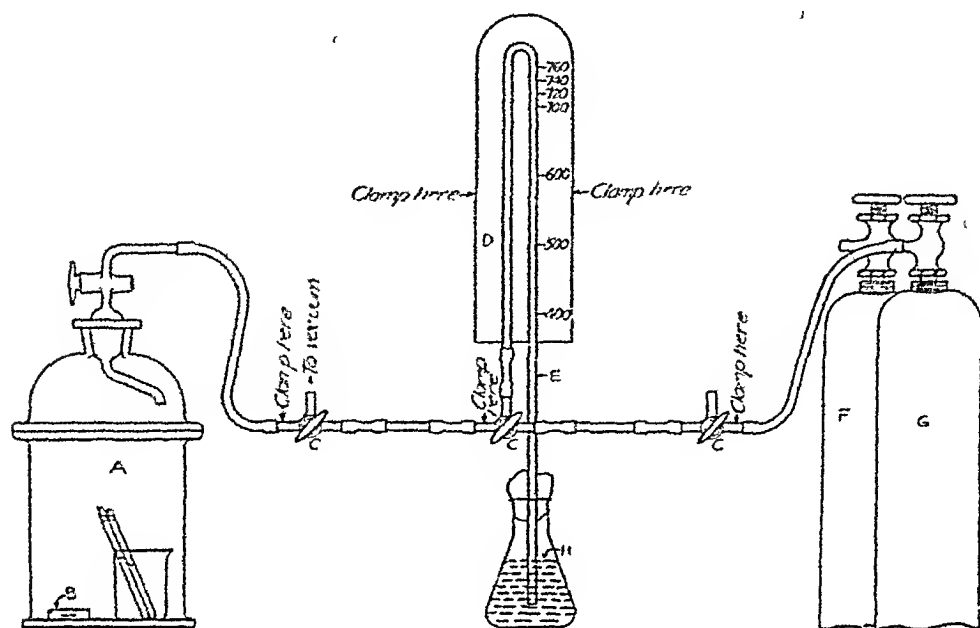
FIG. 224.—THE WEISS-SPAULDING APPARATUS FOR ANAEROBIC CULTURES

A, Hempel desiccator; B, catalyst in jar; C, three-way cocks; D, mirror for reading column of mercury; E. mercury manometer; F, tank of carbon dioxide; G, tank of hydrogen; H, reservoir of mercury.

lesser amount of hydrogen. the vacuum pump need not be used before the hydrogen is admitted and the electric current is turned on.

Method of Cultivating Bacteria under an Increased CO_2 Tension.—This method is used for the isolation of the bovine type of Brucella and often for meningococci and gonococci. The best concentration of CO_2 seems 5 to 10%. An anaerobic jar such as that of Weiss and Spaulding is most convenient to use. Connect the jar with a vacuum pump by means of a three-way stop-cock which is also connected with a mercury manometer and a source of CO_2. Remove sufficient air to decrease the pressure by 5 cm. of mercury and then replace this air with carbon dioxide.

Methods for Fishing Colonies.—Unless well-isolated colonies are found on a plate it is best to replate if possible. Examinations of the colonies may be facilitated by means of a hand lens, dissecting microscope or the low power of the microscope. The colony selected for fishing should be "ringed" with a wax pencil and numbered. Only a part of the colony should be removed for staining, leaving the rest for transfer to sterile media as desired. The fishing is best done with a sharply pointed needle. Colonies in shake tubes are best fished by first expelling

readily set up. Cultures are placed in a sealed Hempel desiccator with 0.5 gm. palladinized asbestos (shredded) and a tube of 2% dextrose in nutrient broth colored with methylene blue (1:50,000). The air is exhausted from the jar by suction and the stop-cock closed. Carbon dioxide is then introduced to a manometer reading of 700 mm. mercury. Hydrogen is then slowly introduced until atmospheric pressure is reached. The stop-cocks are then closed and the jar disconnected for incubation of the cultures.

The *Brown* jar is also quite satisfactory. The catalyst in this jar is platinized asbestos placed on a glass rod and wrapped around with resistance wire through
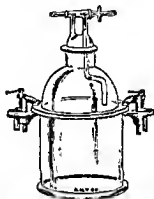
Fig. 222.—Improved Novy Anaerobic Culture Apparatus.
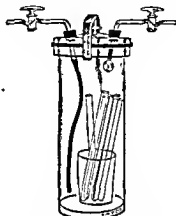
Fig. 223.—Smillie Anaerobic Culture Apparatus.

which is sent a current of electricity to heat the catalyst. The catalyst is enclosed in a netting of fine wire to prevent explosion.

The inoculated cultures (Petri dishes or tubes) are placed within the jar. A piece of "plasticine" (other modeling clays have been found less satisfactory) is rolled out in the form of a thin roll and placed onto the ground edge of the glass jar. The lid is pressed down onto the plasticine so as to make an air-tight joint and is held in place by the screw clamp. If a vacuum pump is available, a large part of the air may be pumped out of the jar. This serves to draw the lid down into place, to minimize the danger of explosion, and to establish anaerobic conditions more quickly. With the partial vacuum in the jar it is connected with the source of hydrogen which is led into the jar under a pressure of only 2 or 3 pounds. The terminals of the heating coil having been connected with an ordinary 110-volt electric light current which has been reduced by passage through a 50-watt light bulb, the current is turned on and allowed to flow for 20 or 30 minutes. If the connections are tight and the hydrogen tank is provided with a reducing valve, the hydrogen may be allowed to flow into the jar throughout this period as fast as it is consumed, a pressure of 2 or 3 pounds being maintained by the reducing

it will be found necessary to reduce the light by partially closing the diaphragm of the microscope to obtain clear definition of unstained bacteria and other cells. Moist preparations are to be dropped into boiling water or into disinfecting solution before being cleaned.

Hanging-drop Preparations.—In the center of a clean thin (No. 1) coverslip 20 mm. square place a small loop of material to be examined (fluid culture or suspension of growth from solid medium). Do not spread the droplet. Place small bits of vaselin or droplets of oil on opposite sides of the concavity of a hollow-ground slide (Fig. 226). Invert the coverslip over the concavity of the slide so that the drop of material hangs beneath

FIG. 226.—HANGING DROP SLIDE

the coverslip without touching the slide into the concavity and with opposite corners of the coverslip protruding beyond the edges of the slide (to facilitate removal of the coverslip when it is to be discarded). Hanging-drop preparations are to be dropped into boiling water or into disinfecting solution before being cleaned. It is not easy to focus the microscope on a hanging drop. Reduce the light with the

FIG. 227.—ZEISS DARK-FIELD ASSEMBLY

diaphragm and find the edge of the drop under the low power objective, then turn the high power objective into place. (It is important that the objectives of the microscope be par-focal and accurately centered.) Having found the edge of the drop under high power, the slide may be moved about carefully while under observation of the technician.

Dark-field Examination.—1. Apparatus necessary (Figs. 227 and 228); strong illuminating lamp such as a small arc lamp or high-power incandescent lamp;

the agar as described under Shake Tube Method. The agar is then cut with a sterile knife or wire close to the desired colony.

MICROSCOPICAL EXAMINATION OF CULTURES AND EXUDATES

1. For smears of cultures on solid media place a small loop of water on the clean slide. With the needle add a minute amount of growth to the water. Mix, spread and dry in the air. From fluid media spread small loop of culture onto the slide; no water need be used. When the smear is perfectly dry "fix" it by passing the slide back and forth through the flame three times (do not overheat) and allow it to cool before staining. For special purposes fixing may be done with methyl alcohol or other reagents.

2. In the case of Petri dishes, remove the cover and place it right side up on the table.

3. Examine the plate with unaided eye or hand lens and ring off selected colonies with wax pencil on bottom of plate.

4. Or select colonies with the aid of the lower part of the microscope (Fig. 225).

5. With a sterile needle carefully remove portions of selected colonies to fresh media and prepare smears for staining. With adherent colonies and especially pneumococci and streptococci, it is sometimes necessary to cut out with a sterile loop a portion of the medium and transfer to broth.

FIG. 225.—FISHING AND TRANSFERRING A COLONY WITH THE AID OF THE MICROSCOPE

(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

6. If the colonies are not well isolated, fish several to broth and prepare another set of plates.

Moist Preparations.—For examining unstained material suspend some of the material in a small drop of water, salt solution, broth or mounting fluid[1] on a clean glass slide. Carefully, so as not to admit air bubbles, place a clean coverslip over the drop. Examine with any of the objectives of the microscope required but

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[1] Mounting fluid, especially useful for examination of fungi, is prepared by mixing equal volumes of glycerol, ammonium hydroxide and alcohol

METHODS OF STAINING BACTERIA

Stains are most conveniently kept in dropping bottles (Fig. 230) or bottles provided with a rubber stopper and nipple with a short dropping pipet attached. The staining is generally done by putting the stain on the slide (Fig. 231), but for some purposes a staining dish is employed (Fig. 232).

Only sufficient stain for covering the smear should be used in the interests of economy. It should not be spread with the tip of the bottle as contamination of the stain may result.

Fig. 230.—Recommended Dropping Bottle

Löffler's Methylene Blue.—For general staining of bacteria and for diphtheria bacilli:

1. Make thin smear of material to be examined on slide.
2. Dry in air and fix with gentle heat.
3. Cover smear with stain and allow to stand for one or two minutes: heat slightly if deep staining is desired.
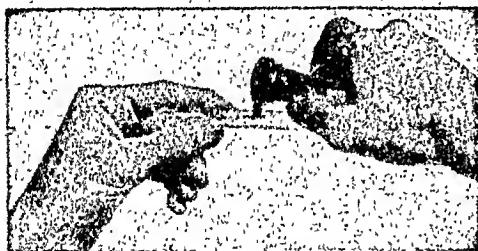
Fig. 231.—Method of Applying Stain to a Slide

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)
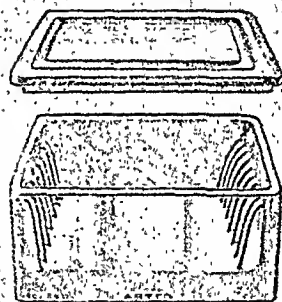
Fig. 232.—Staining Dish

FORMULA OF STAIN

Methylene blue (certified [2]) .... 0.3 gms.
Ethyl alcohol (95%) .... 30.0 c.c.
When dissolved add
Distilled water .... 100 c.c.

4. Wash with tap water, blot and examine with oil-immersion lens.

Methylene blue does not stain very intensely and there is little danger of over-

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[2] The term "certified" as applied to stains indicates a product which has been found satisfactory by the Commission on Standardization of Biological Stains of the Society of American Bacteriologists.

Löffler's methylene blue stain was prepared by adding alkali to the solution. Modern purified samples of methylene blue do not require this. The older preparations contained acid impurities (Conn; Stain Tch., 1929, 4:27. Conn; Biological Stains, Geneva, N. Y., 1929, 67).

funnel stop to be placed in oil-immersion objective to cut out rays which interfere, and a special substage condenser (Fig. 229).

Remove substage condenser from microscope and adjust dark-field condenser in its place.

Fig. 228.—Outfit for Dark-Field Examination for Spirochetes
(From Kolmer, *Chemotherapy and the Treatment of Syphilis*, W. B. Saunders Co.)

3. Insert funnel stop in the oil-immersion objective if one is to be used. (One may use a special oil-immersion lens of N.A. 0.80 without a funnel stop.)

4. Center the apparatus with low-power lens by getting concentric rings on the upper surface of the dark-field condenser and adjusting by means of the three centering screws on edge of the condenser until the rings are parallel with the circle of the microscopic field.

5. Place a small drop of material to be examined on a scrupulously clean slide (1.45 to 1.55 millimeters thick) and cover with clean coverglass (avoid air bubbles).

6. Place large drop of oil on top of condenser and put slide on stage so the oil forms a contact with the under surface of the slide.

Fig. 229.—Substage Condenser for Dark-Field Examination

7. Place oil on top of coverslip and examine with oil-immersion objective. Examination can also be made with low and high power objectives. A darkened room is helpful.

3. Cover smear with 1% aqueous solution of gentian violet.

4. Immediately mix the dye on the slide with 3 to 5 drops of a 5% solution of sodium carbonate in a 0.5% aqueous solution of phenol.

5. Stain for 1 to 2 minutes.

6. Wash quickly with water and cover for one minute with Gram's iodine solution (mix 1 gm. of iodine with 2 gms. potassium iodide; dissolve in a very small amount of water and then add water to 300 c.c.).

7. Wash quickly with water and blot.

8. Decolorize by dropping a mixture of 3 parts acetone (Fig. 233) and 1 part ether until the solution flows colorless from the slide. Equal parts of acetone and 95 per cent alcohol or two parts alcohol and one part of acetone are equally satisfactory. Blot dry.

9. Counterstain for 30 seconds with a 0.5% aqueous solution of safranine.

10. Wash with water, blot and dry. Gram-positive organisms are very dark blue; gram-negative organisms are an orange pink.

Hucker's Modification of the Gram Stain.—

SOLUTION A

Crystal violet (85% dye content)	3.0	gms.
Ethyl alcohol (95%)	20	c.c.

SOLUTION B

Ammonium oxalate	0.8	gm.
Water	80	c.c.

Mix solutions A and B, ordinarily in equal parts. It is sometimes found, however, that this gives so concentrated a stain that gram-negative organisms, such as the gonococcus, do not properly decolorize. To avoid this, solution A may be diluted as much as ten times, and 20 c.c. of the diluted solution mixed with solution B.

1. Stain 15 seconds with the gentian violet solution.

2. Wash in water.

3. Immerse in Gram's iodine solution for 1 minute.

4. Wash in water and blot dry.

5. Decolorize with 95% alcohol or acetone (preferred) for 5-10 seconds with gentle agitation.

6. Drain off alcohol.

7. Cover with counterstain for 10 seconds.

8. Wash, dry, and examine.

Carbolfuchsin (Ziehl-Neelson).—For staining "acid-fast" bacteria (tubercle bacilli, leprosy bacilli, etc.) :

1. Make thin smear on a slide or coverglass. Dry in air and fix with heat.

2. Flood the smear with the following stain and steam gently over the flame about 3 minutes. Do not boil and renew the stain as it evaporates:

staining. It is a good stain to use when studying the morphology of organisms and is sometimes used in the examination of cultures for diphtheria bacilli.

Borax Methylene Blue (Manson).—For staining bacteria in blood smears. Same technic as above:

FORMULA FOR STAIN

Methylene blue	2 gms.
Borax	5 gms.
Distilled water	100 c.c.

Wayson Stain.—For staining diphtheria bacilli and to show nuclear structures of bacteria:

1. The stain is prepared by dissolving 0.2 gm. of fuchsin and 0.75 gm. of

FIG. 233.—METHOD OF DECOLORIZING WITH ACETONE

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, Waverly Press, Inc., Baltimore.)

methylene blue in 20 c.c. of absolute alcohol. Add the dye solution to 200 c.c. of 5% solution of phenol in distilled water. Filter.

2. Stain smears for a few seconds. Wash, blot and dry.

Gram Stain (Burke's Modification).—1. Make thin smear on glass slide or coverglass.

2. Dry in air and fix with gentle heat.

Dissolve the stain in boiling water and filter through filter paper.

4. Wash, dry, and mount.

5. The bacilli may stain uniformly brown or may show at one or both ends a dark blue, round body. True diphtheria bacilli usually show the blue bodies, while the pseudotypes show few if any.

Ljubinsky's Stain for Diphtheria Bacilli.

SOLUTION A

Methyl violet B .... 2.5 gm.
Glacial acetic acid .... 50.0 c.c.
Distilled water .... 950.0 c.c.
Do not filter.

SOLUTION B

Chrysoidin .... 5.6 gm.
Distilled water .... 1000.0 c.c.

This is a differential stain for the polar granules in diphtheria bacilli. It also stains granules in other organisms. The granules should be dark blue or black and the rest of the bacterial body reddish or yellowish. The time of application of the two solutions varies according to the age of the solutions and can be determined only by trial.

Albert's Stain (Laybourn's Modification for Diphtheria Bacilli).

SOLUTION NO. 1

Toluidin blue .... 0.15 gm.
Malachite green .... 0.20 gm.
Glacial acetic acid .... 1.00 c.c.
Alcohol (95%)........................ 2.00 c.c.
Distilled water .... 100.00 c.c.
   Let stand 24 hours, filter.

SOLUTION NO. 2

Iodine crystals .... 2 gm.
Potassium iodide .... 3 gm.
Distilled water .... 300 c.c.

1. Fix smears by heat.
2. Flood with solution No. 1 for 3-5 minutes.
3. Wash in tap water.
4. Flood with solution No. 2 one minute.
5. Wash, blot dry, and examine.
6. The granules stain an intense black, the bars dark green, and the intervening

10% alcoholic solution of basic fuchsin	10 c.c.
5% aqueous solution of phenol	100 c.c.

3. Wash with water and decolorize by dropping acid alcohol on the smear until it flows colorless from the slide:

Concentrated hydrochloric acid	2 c.c.
95% ethyl alcohol	98 c.c.

4. Wash with water and counterstain with Löffler's methylene blue for 1 minute.

5. Wash with water, blot, dry and examine. "Acid-fast" bacilli are pink in a blue background.

Carbolfuchsin (Kinyoun).—1. Stain the fixed smear for 3 minutes without heating with the following:

Basic fuchsin	4 gms.
Phenol crystals	8 gms.
95% ethyl alcohol	20 c.c.
Water	100 c.c.

2. Wash with water and decolorize with acid alcohol as in the Ziehl-Neelson method.

3. Counterstain with Löffler's methylene blue.

Carbolfuchsin for General Staining.—This is an excellent stain for bacteria in general.

1. Fix the smear with heat and cover with water.

2. Add a drop or two of Ziehl-Neelson's stain or cover smear with a 1:10 dilution.

3. Stain for 30 to 60 seconds, wash with water, dry and examine. Bacteria are of a deep pink color. It is important not to overstain.

Neisser's Stain for Diphtheria Bacilli.—1. Prepare smear in usual manner, fix with heat and place in solution No. 1 for 2 or 3 seconds.

SOLUTION NO. 1

Methylene blue (Grubler)	0.1 gm.
Alcohol (95%)	2.0 c.c.
Glacial acetic acid	5.0 c.c.
Water (distilled)	93.0 c.c.

Dissolve the methylene blue in alcohol and add to the acetic acid and water.

2. Wash in tap water.

3. Place in solution No. 2 for 3 to 5 seconds.

SOLUTION NO. 2

Bismarck brown	0.2 gm.
Water (boiling)	100.0 c.c.

evaporation of the alcohol and consequent formation of a colloidal precipitate of the dye complex which is adsorbed by the flagella.

4. Wash with water and examine, or counterstain if desired.

5. Counterstaining is not necessary for routine work. With some bacteria such as typhoid bacilli, the body of the organism is stained only faintly or not at all and the counterstain helps to give a better picture. A satisfactory counterstain is made by diluting borax methylene blue 10 times with distilled water (0.1% methylene blue and 0.5% borax). Allow counterstain to act about 10 minutes. By this means the bacterial bodies become blue and the flagella red.

Casares-Gil Method for Flagella.

MORDANT·

Tannic acid	10	gms.
Aluminum chloride $(Al_2Cl_6 12H_2O)$	18	gms.
Zinc chloride	10	gms.
Rosaniline hydrochloride	1.5	gms.
Alcohol (60%)	40	c.c.

The solids are dissolved in the alcohol by trituration in a mortar, adding 10 c.c. of the alcohol first and then the rest slowly. This alcoholic solution may be kept several years.

1. To make the preparation of bacteria: transfer 1 loopful of growth from an 18-hour agar slant culture of the organism to 2 c.c. of sterile tap water. Incubate this suspension at 37° C. for 10-15 minutes. Transfer one loopful carefully to an absolutely clean slide. Tilt the slide so that the drop runs down, leaving a thin film, or the drop may be drawn out gently with paper. Allow to dry in air.

2. For use, dilute with two parts of distilled water, filter off the precipitate and collect the filtrate on the slide containing the smear of bacteria. Allow this to act 1-2 minutes. A precipitate and metallic sheen should form. Wash with distilled water.

3. Cover preparation with filtered carbolfuchsin and allow to act 1 to 3 minutes. Wash in distilled water. Dry without blotting.

Dorner's Method for Spores.—*Solutions.*—(*a*). Carbolfuchsin (freshly filtered), (*b*) Saturated aqueous solution of nigrosin B (Grübler).

1. Make a heavy suspension of the organism in 2 to 3 drops of distilled water in a small test tube. Use the growth of the culture on an agar slant for this emulsion.

2. Add an equal quantity of freshly filtered carbolfuchsin.

3. Allow the mixture to stand in a boiling water bath 10 to 12 minutes.

4. On a coverslip or slide mix one loopful of the stained preparation with one loopful of a saturated aqueous solution of nigrosin.

5. Smear as thinly as possible and dry rapidly.

The spores are stained red, the bodies of the bacteria are almost colorless and stand out against the dark gray background of nigrosin.

portions light green. The stain is quite specific and will serve to detect diphtheria bacilli when only a few are present.

Flagella Stain (Leifson, Modified).

FORMULA

Potassium alum, 5% aqueous solution ...... 10 c.c.
Tannic acid, 2% aqueous solution .... 10 c.c.
Basic fuchsin (dye content 100%) in 1%
  solution in 95% alcohol .... 10 c.c.
The solutions are mixed in the order given.

The tannic acid solution usually develops molds and for this reason will not keep. A mixture of solutions (1) and (2) seems to keep and also seems to be stable. Rosaniline hydrochloride may be used instead of the basic fuchsin. The concentration given is based on the pure dye and since the commercial dyes are seldom pure (75-90%) they must be used in correspondingly higher concentration. For example, a dye labeled "85% pure" should be used in 1.18% concentration and a "75% dye" in 1.33% concentration.

The flagella stain is stable for several days provided it is kept well stoppered. A new mixture need only be made about once a week. The precipitate which forms is of no consequence. In old stains it is best to use only the supernatant.

1. Use only young broth or moist agar cultures. Both cultures are centrifuged, the supernatant fluid poured off, and the bacteria carefully suspended in distilled water. The suspension is again centrifuged, the supernatant poured off, and the bacteria resuspended in distilled water. Smears from agar slants are made by suspending some of the growth from the bottom or middle of the slant in distilled water. The growth at the top of the slant should not be used. Place a large loopful of the suspension on the end of a prepared slide. Tilt the slide to cause the suspension to flow down the slide. If it does not flow readily the slide is greasy and should be discarded. Allow to dry in air at room temperature. No fixing is necessary.

2. Slides should be free from scratches and absolutely grease-free. The best method of cleaning the slides is to put them in a hot solution of potassium dichromate in concentrated sulphuric acid for a few hours. They are then thoroughly washed with distilled water and allowed to drain and dry in air. They should never be wiped with a cloth. Just before use the slide should be flamed strongly (film side towards the flame), and a wax pencil line made along the edges of the slide leaving about ½ inch free for handling. The wax pencil lines serve to confine the stain and allows it to be heaped up on the slide.

3. With a capillary (Pasteur) pipet place 0.8 to 1 c.c. of the stain on the prepared slide. The slide should be level or on a very slight incline. It is important that the stain be heaped up on the slide. Allow the stain to remain on the slide for about 10 minutes. With a high room temperature and much circulation of air the staining may be complete in about 5 minutes. In a cold room with no circulation the staining may take 15 minutes. The staining of the flagella depends upon the

STAIN

Carbolic acid (2% aq. sol.)	100.0 c.c.
Acetic acid (1% aq. sol.)	1.0 c.c.
Lactic acid (conc.)	0.5 c.c.
Carbolfuchsin	1.0 c.c.
Basic fuchsin (sat. alc. sol.)	1.0 c.c.

3. Wash in water and dry.

4. This method is applicable to cultures only; not to exudates.

India Ink Method for Capsules.—On a clean slide mix the culture with a loop of India ink. Spread to a thin film as in making blood smears. Allow to dry; fix. Stain for one minute with methylene blue or for a few seconds with basic fuchsin. Wash with water, dry and examine. Only the bacterial bodies are stained, the capsules appearing as clear spaces about the bacteria.

Fontana's Stain for Spirochetes (Tribondeau).—For staining spirochetes in smear preparations of chancres and organs:

SOLUTIONS

(1) Fixing solution

Acetic acid, glacial	1 c.c.
Formalin	20 c.c.
Water	100 c.c.

(2) Mordant

Tannic acid	5 gm.
Carbolic acid	1 gm.
Water	100 c.c.

(3) Silver solution

Silver nitrate	1 gm.
Water	20 c.c.

Dissolve the silver nitrate, then add drop by drop a 10% ammonia solution until the precipitate redissolves. The solution at this stage should be slightly opalescent; if clear, a few drops of silver nitrate solution should be added to give a faint turbidity.

1. Prepare a smear and allow to dry; treat with the formalin fixative for 1 to 2 minutes.

2. Wash thoroughly with absolute alcohol; in case of smears from organs, the fat should be removed by the application of ether after the alcohol and a final application of alcohol.

3. Pour on the tannin mordant and heat till steam arises; continue for half a minute.

4. Wash thoroughly in tap water, then in distilled water.

5. Stain with the silver solution for a few seconds in the cold, then pour off and add fresh silver solution and heat till steam rises for a quarter of a minute.

Moeller's Method for Spores.—1. Coverslips are prepared as usual and fixed in the flame.

2. Wash in chloroform for 2 minutes.

3. Wash in water.

4. Cover with 5% chromic acid one-half to 2 minutes.

5. Wash in water. Invert and float coverslip on carbolfuchsin solution in a small porcelain dish and heat gently with a flame until it steams; continue this for 3 to 5 minutes. (This step can also be done by covering the coverglass with carbol-fuchsin and holding over flame.)

6. Decolorize with 5% sulphuric acid 5 to 10 seconds.

7. Wash in water.

8. Stain with aqueous methylene blue one-half to 1 minute. By this method spores will be stained red, the body blue.

Gram's Stain for Capsules.—Thin smears stained by the gram method as described are frequently satisfactory for capsules.

Welch's Method for Capsules.—1. Cover film with glacial acetic acid for few seconds.

2. Drain and replace with aniline gentian violet. Drain and again replace with aniline gentian violet. Repeat until all acid has been replaced by gentian violet solution.

3. Wash in a 1 or 2% solution of sodium chloride and mount in same. Do not use water at any stage.

4. The capsule stains pale violet.

Hiss's Copper Sulphate Method for Capsules.—1. Grow organisms in ascitic fluid or serum medium or mix with drop of serum and from this mixture prepare smears.

2. Air dry and fix with heat.

3. Cover preparation with 5% water solution of gentian violet and heat for few seconds until steam arises (5 c.c. of saturated alcoholic solution of gentian violet to 95 c.c. of distilled water).

4. Wash dye off with 20% solution of copper sulphate crystals.

5. Blot (do not wash) and dry thoroughly.

6. By this method permanent preparations are obtained, the capsules appearing with faint blue halos around dark purple cell bodies.

Huntoon's Method for Capsules.—1. Mix the organisms with a small drop of nutrose solution on a slide and spread the film; dry in air (do not fix).

<div align="center">NUTROSE SOLUTION</div>

Nutrose (sodium caseinate)	3 gm.
Water	100 c.c.

Cook the solution for one hour in Arnold sterilizer and add 0.5% carbolic acid. Place in tubes without filtering.

2. Cover with stain for 30 seconds.

2. Wash in water. Place in 40% formalin for a few seconds (bright red color fades to a clear rose). Wash in water.

3. Counterstain in saturated water solution of picric acid for 3-5 minutes (until section assumes a purplish-yellow color). Wash in water.

4. Differentiate in 95% alcohol (red appears and some is washed out; some picric acid is washed out). Wash in water.

5. Stain in Sterling's gentian violet for 5 or more minutes:

> Gentian violet .... 5 gm.
> Alcohol (95%) .... 10 c.c.

Grind in a mortar and add:

> Aniline oil .... 2 c.c.
> Water (distilled) .... 88 c.c.
>   Let stand for 1 or 2 days and filter.

6. Wash in water and place in Gram's iodine solution for 5 minutes. Blot dry without washing.

7. Place in aniline oil and xylol (equal parts) until no more color comes away. Place in two changes of xylol. Mount in Canada balsam.

8. Gram-negative organisms stain red; gram-positive organisms, blue; tissues stain in shades of red to purple.

Gram-Weigert Method for Paraffin Sections.—1. Fixation in Zenker's solution is preferred.

2. Stain sections lightly in alum-hematoxylin. Wash in running water.

3. Four per cent aqueous solution eosin soluble in water, 5 minutes to ½ hour. Wash in water.

4. Aniline methyl violet ½ to 1 hour. Wash off with water.

5. Lugol's solution, 1 to 2 minutes. Wash off with water.

6. Blot with filter paper and dehydrate and clear in several changes of aniline and xylol, equal parts, or in aniline oil alone. Wash off with xylol. Mount in xylol-colophonium.

Pappenheimer's Method for Gram-positive and Gram-negative Organisms.
—1. Zenker fixation preferred; paraffin sections should be 5 microns or less.

2. Stirling's gentian violet for 5 minutes; Gram's iodine for 1 minute. Aniline oil, or aniline oil, xylol. Decolorize to pale violet.

3. Absolute alcohol, for a few minutes only.

4. Wash with distilled water.

5. Aqueous safranine ½% for 30 seconds.

6. Distilled water and blot.

7. Absolute alcohol: few minutes only. Clean in xylol.

Brown and Brenn Stain for Gram-positive and Gram-negative Organisms.
—1. Stain in freshly filtered alum-hematoxylin (Harris) for 2 to 5 minutes.

2. Wash in acid alcohol (3% HCl in 95% alcohol) until light pink.

6. The slide, which should now be a distinct brown color, is washed in distilled water, blotted and dried. The spirochetes should, if the method has been properly carried out, be of a blackish or brown color. The film should be mounted in Canada balsam, since when examined without a coverslip the cedar-wood oil discolors the spirochetes.

India Ink Method for Spirochetes (Burri).—On one end of a slide mix an equal amount of secretion from a chancre or other material with India ink free of artefacts. Smear as described for making blood smears. Allow to dry and examine. The spirochetes appear as white spirals on a black background.

Tunnicliff Method for Spirochetes and Fusiform Bacilli.—1. Make a thin smear of the material on a slide. Fix with heat.

2. Cover with carbol crystal violet, 30 seconds. (Sat. alc. sol. crystal violet, 10 c.c.; 5% aqueous phenol sol. 90 c.c.)

3. Wash with water.

4. Cover with Lugol's iodine solution, 30 seconds.

5. Wash with water.

6. Cover with safranine, 30 seconds.

7. Wash with water.

Spirochetes and fusiform bacilli stain purplish black. Large bacterial forms are often granular. Capsules may be demonstrated by this method occasionally.

METHODS OF STAINING BACTERIA IN TISSUES

Preparation of Tissue Section.—1. Obtain tissue as soon as possible after death to prevent postmortem changes.

2. Place blocks not larger than one-quarter by one-eighth inch in Zenker's fluid for 3-12 hours.

3. Wash in water for several hours.

4. Place for 24 hours in each of the following alcohols in succession: 30, 60, 90% and absolute.

5. Place in cedar oil or xylol until translucent.

6. Place in equal parts of cedar oil or xylol and paraffin at 37° C. for 2 hours.

7. Place in paraffin at 52° C. for 2 hours in each ol 2 baths.

8. Box and cut sections of 3 to 6 micra.

9. Dry sections in incubator for about 24 hours or over night.

10. Remove the paraffin by placing the slides in xylol and then in absolute alcohol. Repeat until all paraffin is removed and then place sections in water (no clouding denotes removal of paraffin).

Goodpasture's Stain.—1. Place prepared section in the following stain for 10 to 30 minutes:

Alcohol	100.00 c.c.
Basic luchsin	0.59 gm.
Aniline oil	1.00 c.c.
Phenol (crystals)	1.00 gm.

Second Method for Staining Tubercle Bacilli in Tissue.—Fix tissue in formol solution or other fixative. After sections have been cut and the paraffin removed, pass them down to water. Stain in carbolfuchsin 3 hours at room temperature.

<div align="center">STOCK SOLUTION</div>

Basic fuchsin	10 gms.
Absolute alcohol	100 c.c.

Use for staining

Stock solution	10 c.c.
Phenol 5%	90 c.c.

Treat slides with acid alcohol (alcohol 80%, 98 c.c., hydrochloric acid. 2 c.c.) until almost decolorized. Wash in distilled water. Blot. Wash in water. Stain in Harris's hematoxylin, 1 minute. Wash in water. Acid alcohol, 2 to 4 seconds. Wash in water. Blot. Wash in aniline oil. Treat with one third aniline oil and two-thirds xylol. Clear in xylol and mount in balsam. *Balsam must be neutral or the slides will fade in time.*

Mallory's Method for Actinomyces.—Stain deeply in saturated aqueous eosin 10 minutes. Wash in water. Aniline gentian violet from 2 to 5 minutes. Wash in normal saline solution. Weigert's iodine solution (Iodine 1, KI 2, and water 100 parts) 1 minute. Wash in water and blot. Clear in aniline oil. Xylol several changes. Mount in balsam.

<div align="center">METHODS FOR STUDYING BIOLOGICAL PROPERTIES OF BACTERIA</div>

Study of Colonies.—*Surface colonies* on agar media are often of considerable value in identification. Such colonies of different bacteria may differ considerably and an experienced bacteriologist can often make a tentative diagnosis by the study of the surface colonies. This is especially true where the bacteria are planted on special media and exhibit peculiar colonial forms. The features of colonies which should be noted especially are whether the colonies are: dry or moist, mucoid, rough or smooth, wrinkled, translucent or opaque, shape of edges, flat or elevated, spreading, and color. By means of a hand lens or the low power of the microscope more details are brought out.

Deep colonies usually are less characteristic than surface colonies, except in special media. In blood agar the deep colonies are especially informative and are the basis for the differentiation of the streptococci. Deep colonies are best studied with the lower power of the microscope, or better, by unscrewing the lower half of the low power lens of the microscope a magnification is obtained which seems just right.

Appearance in Broth.—The growth in broth is often of diagnostic value. The growth may be homogeneous, granular, flocculent, or mucoid. A pellicle may form on the surface, and a precipitate on the bottom.

3. Wash in ammonia water (1 c.c. of aqua ammonia in 100 c.c. water) until blue. Wash in water.

4. In a small vial mix 5 drops of 5% aqueous solution of sodium bicarbonate (containing also 0.5% phenol as preservative) with about 0.75 c.c. of 1% aqueous solution of gentian violet. Immediately pour the mixture onto the slide and stain for 2 minutes. Wash quickly with water.

5. Cover with iodine solution (iodine 1 gm., potassium iodide 2 gm., water 300 c.c.) for 1 minute.

6. Wash with water. Blot.

7. Decolorize in 1 part of ether plus 3 parts of acetone, dropping it onto the slide until no more color comes off. Blot.

8. Stain for 5 minutes with rosaniline hydrochloride (0.005 gm. per 100 c.c. water). Wash in water. Blot but do not allow the section to dry.

9. Pass through acetone. Decolorize and differentiate by dropping over the section a solution of 0.1 gm. of picric acid in 100 c.c. of acetone until the section becomes yellowish-pink. This is the most critical stage of the process and should be carried out by holding the slide over a white plate or dish. Most of the rosaniline should be decolorized from the tissue but the gram-negative bacteria should remain red. Pass successively through acetone, equal parts of acetone and xylol, and xylol. After clearing in xylol mount in balsam. (Beginning with step 5, it is best to work with only one slide at a time.) Cell nuclei should be stained dark reddish-brown; cytoplasm yellowish; gram-positive bacteria deep violet or almost black; gram-negative bacteria bright red. Leukocytes generally stand out plainly with a dusky yellowish cytoplasm. Basophilic granules stain red. Red blood cells may be a yellow or red depending upon the degree of decolorization in picric acid. Cartilage stains pink. Striated muscle and fibrin generally stain yellow but may retain more or less of the red stain.

Mallory and Wright's Method for Tubercle Bacilli.—*Paraffin Sections.*—
1. Stain in carbolfuchsin solution hot for 5 minutes (or better, cold, for 24 hours). Wash in water. Decolorize and counterstain in Gabbet's methylene blue-sulphuric acid mixture for 1 minute:

Methylene blue	2 gms.
Sulphuric acid	25 c.c.
Water	75 c.c.

2. Wash in water. Dehydrate in absolute alcohol. Clear in xylol. Mount in balsam.

Celloidin Sections.—Stain lightly in alum hematoxylin. Wash in water. Dehydrate in 95% alcohol. Attach the slide by ether vapor. Stain with steaming carbolfuchsin 2 to 5 minutes. Wash with water. Wash with Orth's acid alcohol (alcohol 90%, 99 c.c. conc. hydrochloric acid, 1 c.c.) ½ to 1 minute. Wash in water several changes. Treat with 95% alcohol until red color is entirely gone. Blot and cover with xylol until clear. Mount in balsam.

carbon dioxide and hydrogen. The ratio of the carbon dioxide and hydrogen which is produced is of diagnostic value in differentiating such groups of bacteria as aerobacter and eschericia. The analysis of the gas may be done in a Smith fermentation tube by absorbing the carbon dioxide with sodium hydroxide. The residual gas is mostly hydrogen. Some years ago this test was commonly used but now it has been largely abandoned.

Action on Organic Acids.—Some organic acids are used for differentiation of bacteria. Most of these are used in synthetic media as the sole energy source. Among acids thus used may be mentioned citric, malonic and tartaric. .

Production of Indol.—This seems to be a fundamental and important property of bacteria. Indol is produced from tryptophane and any medium used for this test must contain tryptophane in appreciable quantities. The following tests are recommended:

Oxalic Acid Paper Test for Indol (Holman and Gonzales).—Soak filter paper in saturated oxalic acid solution. Dry and cut into strips. Hang a strip of the paper in the form of a loop over the medium in a culture tube, securing the ends of the paper between the cotton plug and the mouth of the tube. Indol is shown by the development of a pink color on the paper during the growth of the culture. The paper must not be allowed to become wet.

Ehrlich Indol Test.

REAGENT

Paradimethylaminobenzaldehyde .... 2 gms.
Ethyl alcohol (95%) .... 190 c.c.
Hydrochloric acid (conc.) .... 40 c.c.

Add about 1 c.c. of ether to the peptone water and shake. Allow ether to rise to the top and form a layer. Add about one-half c.c. of reagent so that it forms a layer between the medium and the ether. A red color forms at the junction and spreads into the ether layer.

Reduction of Nitrates.—Reduction of nitrates to nitrites demonstrates the reducing ability of bacteria. This reaction is not of very great diagnostic value except in isolated instances. The reaction may be demonstrated in either agar or fluid media.

Test for Nitrites.

SOLUTION A

Sulphanilic acid .... 4 gms.
N/5 acetic acid (Sp. gr. 1.041) .... 500 c.c.

SOLUTION B

Alpha-naphthylamine acetate .... 2.5 gms.
N/5 acetic acid (Sp. gr. 1.041) .... 500 c.c.
        Filter through washed absorbent cotton.

Production of Pigment.—Many bacteria produce very intense pigments which are characteristic and of considerable value in diagnosis. Such pigments are produced more abundantly in some media than in others. Löffler slants are very good for demonstrating pigmentation. Pigment production is generally best under aerobic conditions and may fail altogether in the absence of oxygen, as in the case of *B. pyocyaneus* and *Serratia marcescens*. In many instances more pigment is produced at lower temperatures so that it is advisable to incubate at temperatures of about 20° C. (gelatin incubator) as well as at 37° C.

Liquefaction of Gelatin and Coagulated Serum.—Proteolytic activity of bacteria is usually studied by inoculation into gelatin or on coagulated serum slants. *Gelatin liquefaction may be studied by incubating the inoculated gelatin at 37° C.* for one or more days and then placing it in cold water to see if it will solidify. A better method is to stab the solid gelatin medium and incubate at 20-22° C. By this means the type of liquefaction may be observed and more information is, thus obtained.

Reaction in Milk.—Milk has long been a favorite with bacteriologists. The milk may be coagulated due to acid production by the fermentation of the lactose or by the action of rennin-like enzymes. The coagulum may be digested due to proteolytic enzymes. The completion of this latter reaction may take several weeks.

Action on Carbohydrates.—A large variety of carbohydrates are used in the study of bacteria. Many of these are unstable and cannot be sterilized in the medium in the autoclave without undergoing some hydrolysis. Such carbohydrates can often be sterilized unchanged in 10-20% solution in distilled water and then added aseptically to the medium. Such concentrated sugar solutions are best kept after sterilization in tubes under a vaselin seal (the vaselin being sterilized in the oven at 160° C. for 2 hours before being added to sugar solution), or in tubes with a rubber cap to prevent evaporation of the water. Very delicate carbohydrates must be sterilized by filtration through a sterile bacteriological filter. The carbohydrates are generally added to media in 0.5% or 1% concentration.

In the study of streptococci and allied bacteria, when the final acidity attained is of diagnostic importance it is best not to add the indicator to the medium. At the end of the incubation period (5 to 7 days) a small amount of the culture is removed and the pH determined by the method of Brown or other satisfactory methods. Where it is only necessary to determine whether acid is produced or not, the indicator may be added to the medium *before* sterilization.

To demonstrate gas production shake or stab cultures in agar media are often used, or liquid media covered with a petrolatum seal. The Smith type of fermentation tube may be used but by far the most common is the Durham tube. A small glass tube is inverted in the tube of medium. When the medium is autoclaved the inverted tube becomes *filled with the medium*. Any gas produced is trapped in the inverted tube.

The amount of gas produced may vary with the conditions of the culture and the nature of the medium. Gas production may be completely inhibited by nitrates or nitrites in as low as 0.1% concentration. The gas produced is usually mainly

degree of acidity is made with methyl red indicator by adding a few drops of a 0.04% solution of methyl red in 60% alcohol. A red color (acid) is called a positive test; a yellow color (alkaline) is a negative test.

Ferric Chloride Test for Hydrolysis of Sodium Hippurate.—Reagent: 12 gms. of ferric chloride ($FeCl_3 6H_2O$) dissolved in 100 c.c. of 2% hydrochloric acid in water. Transfer 0.8 c.c. of culture in sodium hippurate broth to a small test tube (Wassermann tube) and add 0.2 c.c. of the reagent. Mix immediately and observe after 10 to 15 minutes. A permanent precipitate indicates the presence of benzoic acid (positive hydrolysis).

Since sodium hippurate is first precipitated and later redissolved by the amount of reagent specified and since benzoic acid is also redissolved by a greater excess of the reagent, it is necessary to have the reagent and the medium balanced and to measure the amounts used in the test quite accurately. A control test of the sterile medium should always be made. If the culture is quite turbid so as to confuse the reading of the result, it should be centrifuged and the clear supernatant used for the test.

Solubility Test for Pneumococci with Sodium Desoxycholate.—Reagent:

Sodium desoxycholate	10 gms.
Alcohol	10 c.c.
Water	90 c.c.

Method.—To 1 c.c. of broth culture add 2 drops of reagent. Dissolution of pneumococci generally occurs in less than 5 minutes. Bile is commonly used for this test but the action is much slower and the results are much less clear-cut than with sodium desoxycholate.

Hemolysin Test.—For differentiation of human and bovine types of hemolytic streptococci.

With sterile capillary pipet transfer to small serological test tubes 10 to 15 drops of the broth cultures. To each tube add an equal volume of 5% suspension in salt solution of washed rabbit blood cells. Incubate at 37° C. for 2 hours. The amount of laking of the blood cells is indicated by negative. one plus, two plus, three plus and four plus.

Action on Blood Agar.—Bacterial colonies (deep) in blood agar are of at least three types. The designations alpha (a), beta (β) and gamma (γ) of Smith and Brown have gained general acceptance. Any types of bacteria may produce these appearances but the cause may be different. Gram-negative bacilli producing the alpha type of colony do so because of the hydrogen sulphide which is produced, while the alpha type of streptococci. or pneumococci, produces no hydrogen sulphide but changes the hemoglobin to hematin which is oxidized to a greenish or brown substance. The alpha type of colony in the case of the cocci is characterized by an immediate zone of unhemolyzed blood cells, usually colored either green or brown. Outside of this zone is a partially clear area. The beta type of colony is the frankly hemolytic type. Recently various bacteria have been found to produce double or even triple zones of hemolysis, especially following re-

To 10 c.c. of culture add 0.2 c.c. of Solution A. Add Solution B drop by drop until a red color appears. In the presence of nitrites a red azo-compound is formed. The reagents must be added in the order given.

Cholera Red Reaction.—For the identification of *V. cholera*. Culture in peptone water for 2 or 3 days. Add a few drops of concentrated sulphuric acid. A positive reaction is indicated by the development of a purplish pink color (a nitrose-indole reaction).

Acetyl-Methyl-Carbinol Test (Voges-Proskauer Reaction, Leifson).—This test is extensively used for the differentiation of aerobacter and escherichia. The culture may be tested after 1 or more days of incubation. Reagent: Dissolve 1 gm. of $CuSO_4.5H_2O$ in 10 c.c. of distilled water and when dissolved mix with 40 c.c. of concentrated ammonia water. Add 950 c.c. of 10% aqueous solution of sodium hydroxide. Add an equal volume of the reagent to the culture and shake thoroughly. A positive reaction is indicated by the development in 10 to 20 minutes of a red color (not to be confused with the violet tint of the biuret reaction which also occurs).

Production of Hydrogen Sulphide.—The production of hydrogen sulphide is of some value in differentiating bacteria but the test must be used with caution. Media for this test must be carefully standardized to get consistent results. By adding cystine or sodium thiosulphate to a medium almost any type of bacteria will produce hydrogen sulphide. The concentration of agar has also a considerable effect. Addition of meat infusion increases hydrogen sulphide production. The usual test for hydrogen sulphide is by means of lead acetate or ferric citrate, which are incorporated into agar media. Blood may also be used and it turns a strong green color in the presence of hydrogen sulphide and oxygen.

Analysis of Gas Formed by Bacteria.—*Carbon Dioxide.*—1. For the estimation both qualitatively and roughly quantitatively of carbon dioxide produced by bacteria, cultures are grown in fermentation tubes containing sugar-free broth to which 1% of pure dextrose, lactose, saccharose, or other sugars has been added.

2. The tubes are incubated until the column of gas formed in the closed arm no longer increases (24 to 48 hours). The level of the fluid in the closed arm is then accurately marked and the column of gas measured.

3. The bulb of the fermentation tube is then completely filled with N/5 sodium hydroxide solution, the mouth closed with a clean rubber stopper, and the bulb inverted several times in order to mix the gas with the alkali. The tube is then again placed in the upright position, allowing the gas remaining to collect in the closed arm. The gas lost may be roughly estimated and considered as being carbon dioxide.

Hydrogen.—The gas remaining, after removal of the carbon dioxide in the preceding may be estimated as hydrogen. When allowed to collect near the mouth, further evidence of its being hydrogen may be gained by exploding it with a lighted match.

Methyl Red Test.—For differentiating aerobacter and escherichia. The bacteria are cultivated for several (4) days in the special (V-P-M-R) medium. A test for

TABLE I

GRAM POSITIVE COCCI WHICH GROW ON BLOOD AGAR

Type of Colony	Morphology	Media for Subculturing	Identification Methods	Tentative Classification
Large, opaque, circular, smooth edges.	Grouped irregularly. Not encapsulated.	Plain agar	Pigment: None / Orange / Lemon	*Staphylococcus Albus* / *Staphylococcus Aureus* / *Staphylococcus Citreus*
Glistening, circular, smooth edges, viscid.	Grouped in tetrads encapsulated.	Plain agar		*M. tetragenus.*
Moist, often viscid, smooth edges 1 mm. or more in diameter, green zone surrounds colony.	Occurs in *pairs, sometimes chains.* Often lancet shaped, capsules usually demonstrable.	Blood infusion broth or Rosenow's brain broth, and Hiss serum water + inulin.	Soluble in bile. Ferments inulin.	Pneumococcus.
Small, green zone surrounds colony.	Occurs in pairs and chains. No capsules.	Blood infusion broth or Rosenow's brain broth, and Hiss serum water + inulin.	Not soluble in bile. Does not ferment inulin.	Streptococcus alpha type. (viridans)
Small, surrounded by a hazy, colorless zone.	Occurs in pairs and chains. No capsules.	as above	as above	Streptococcus alpha prime type.
Small, surrounded by a clear, colorless zone.	Occurs in pairs and chains. No capsules.	as above	as above	Streptococcus beta type. (hemolyticus)
Vary greatly in size. No change in the media surrounding colony.	Occurs in pairs and chains. No capsules.	as above	as above	Streptococcus gamma type. (indifferens)

frigeration of the blood plates. **Differentiation** of the various types of colonies should always be done by means **of the low power** of the microscope after incubation for one or two days **followed by refrigeration** for one day. The blood agar plate constitutes one of the most **serviceable** of the culture media; the size and shape of the colonies frequently **lend much aid** in the identification of many pathogenic species as briefly summarized in tables 1, 2 and 3.

TABLE III

GRAM NEGATIVE RODS WHICH GROW ON BLOOD AGAR PLATES

Type of Colony	Morphology	Media for Subculturing Colony	Tentative Classification
Large, opaque; smooth or irregular edges; smooth or rough surface	Short, plump (sometimes encapsulated)	Plain agar	Coli-Typhoid-Paratyphoid-dysentery
Large, opaque, viscid, smooth edges	Short, plump, encapsulated	Plain agar	Klebsella group
Large, opaque, spreading, dirty, discoloration of media, disagreeable odor	Short, plump	Plain agar	No pigment: B. proteus
			Green Pigment: B. pyocyaneus
Very small, moist	Very tiny	Chocolate agar	Hemophilus group
Small, yellow, round, opaque	Small, stains irregularly	Glycerine—veal agar	B. mallei
Small, translucent, irregular borders	Ovoid, bi-polar, pleomorphic	Plain agar	Pasteurella group
Small, round, regular borders	Small, oval	Plain or blood agar	Br. melitensis

TABLE II

GRAM POSITIVE RODS WHICH GROW ON BLOOD AGAR

Type of Colony	Morphology	Spores	Motility	Identification Methods	Tentative Classification
Large with irregular margins.	Straight, slender, sometimes chains.	Spores formed	Non motile	Pathogenic for mice	*B. anthracis*
			Non-motile	Not pathogenic for mice	Saprophytic organism
			Motile		Saprophytic organism
Small	Pleomorphic; often contain metachromic granules.	Not formed	Non-motile	Toxic for Guinea pigs	*C. diphtheriae*
				Not toxic for Guinea pigs	Diphtheroids

2. *Carbuncles* are larger and more severe and are produced by *Streptococcus hemolyticus*, *Staphylococcus aureus* or a mixture of the two.

3. Chronic discharging furuncles and carbuncles may show additional organisms of secondary infection or contamination listed below.

4. *Anthrax* of the skin usually occurs as a severe furuncle with lymphadenitis called the "malignant pustule" caused by *B. anthracis*.

5. Furuncles of the skin may occur in infections by pathogenic fungi as in actinomycosis, blastomycosis and sporotrichosis.

Method.—1. Prepare thin smears of pus or serous fluid collected as described on page 380 on slides and stain by the method of Gram: examine:

Gram-positive
$\begin{cases} \text{Staphylococci} \\ \text{Streptococci} \\ \textit{B. anthracis} \\ \textit{B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)} \\ \textit{B. subtilis} \end{cases}$

Gram-negative
$\begin{cases} \textit{B. pyocyaneus (Pseudomonas aeruginosa)} \\ \textit{B. coli (Escherichia coli)} \\ \textit{B. proteus-vulgaris} \end{cases}$

. 2. Inoculate a tube of glucose hormone broth and a blood agar slant. If mixed infection is suspected prepare a blood agar plate by surface streak method.

3. Incubate 24 to 48 hours. Examine colonies and prepare smears; stain by Gram method. Employ special methods described for identification of organisms.

BACTERIOLOGICAL EXAMINATION OF INFECTED WOUNDS

Principles.—1. Traumatic wounds are likely to become infected with both aerobic and anaerobic organisms from the skin, clothing, foreign bodies, etc. There is usually a latent period of 12 to 24 hours during which but few bacteria are found.

2. Gangrene is usually caused by one or more of the pathogenic anaerobic bacilli in association with aerobic organisms and is separately described on page 417.

3. In the ordinary infected wound aerobic methods of examination are usually sufficient. If, however, gangrene or tetanus infection is suspected anaerobic culture methods should be included as described under Bacteriological Examination of Gangrene. The areas at which smears and cultures are made should always be those in which bacteria are most likely to be present in large numbers as around foreign bodies, necrotic bone and deep in the sinuses and crevices of the wound.

Method.—1. Pus or secretions may be collected on sterile swabs and sent to the laboratory or smears and cultures may be directly prepared.

CHAPTER XIX

DIAGNOSTIC BACTERIOLOGICAL METHODS

REGIONAL DISTRIBUTION OF PATHOGENIC ORGANISMS

1. When material is submitted for bacteriological examination its source should be stated, as this information may indicate the probable bacteriological findings and guide the method of examination; for example, pus from an acute furuncle or "boil" usually shows a staphylococcus in pure culture. It is therefore helpful in bacteriological diagnosis to keep in mind the regional distribution of the pathogenic organisms, although in smears and cultures of the skin and mucous membranes open to air contamination, various nonpathogenic bacteria may be encountered which are not included herewith except such common ones as B. subtilis, B. proteus-vulgaris and the like.

2. No attempt has been made to include all the organisms found and described in the different locations listed. For example, at least forty-six different ones have been found in the saliva and even a larger number in the feces, but the majority of them are little or no importance. For the identification of these the worker is referred to Bergey's Manual of Determinative Bacteriology.

3. When material like feces containing many different bacteria is submitted for bacteriological examination, the specimen should be accompanied by a request designating the particular organism or organisms to be examined for.

4. Since a smear of material or its culture stained by the Gram method at once yields very useful information, it has proved helpful to arrange the regional distribution of organisms on the basis of this differential stain.

5. Animal parasites (protozoa and metazoa) are not included but are given elsewhere in the sections devoted to the examination of feces, sputum, etc.

6. The new nomenclature of the American Society of Bacteriologists is given as well as the older names for the various micro-organisms for the assistance this may give in gaining familiarity with the newer terminology.

7. A few of the pathogenic micro-organisms may be identified by stained smears alone (gonococci, diphtheria bacilli, tubercle bacilli, etc.) but the majority require a study of cultures and various biological characteristics described on succeeding pages.

BACTERIOLOGICAL EXAMINATION OF PUS FROM FURUNCLES AND CARBUNCLES

Principles.—1. Regardless of location (skin, eyelids, external auditory canal, etc.) furuncles (abscesses or "boils") are usually due to infection by Staphylococcus aureus or albus; "stitch" abscesses and pimples are usually caused by the latter.

BACTERIOLOGICAL EXAMINATION OF GANGRENE OF WOUNDS AND METHODS FOR THE IDENTIFICATION OF PATHOGENIC AND NONPATHOGENIC ANAEROBIC BACILLI

Principles.—1. Gangrene of wounds is usually due to infection with one or more of the pathogenic, anaerobic, spore forming bacilli.

2. As a general rule secondary infection with staphylococci, streptococci, or any of the aerobic organisms mentioned under the Bacteriological Examination of Infected Wounds are usually present.

3. Gangrene due to vascular occlusion as in Buerger's disease, diabetes mellitus, etc., usually shows secondary infection or contamination with aerobic organisms.

4. Gangrene of infected wounds may be predominantly gaseous or phlegmonous. The large, gram-positive, spore forming and anaerobic bacilli responsible for the infection of wounds are usually the result of contamination, as their natural habitat is the intestinal tract of man and animals.

5. Any one or more of the following gram-positive bacilli may be found in smears and cultures:

> (a) B. Welchii (Clostridium welchii) or B. perfringens primarily responsible for "gas" gangrene
>
> (b) B. oedematis-maligni (Clostridium oedematis-maligni) or Vibrion septique primarily responsible for "phlegmonous" gangrene
>
> (c) B. oedematiens (Cl. oedematiens) in "phlegmonous" gangrene
>
> (d) B. histolyticus (Cl. histolyticus)
>
> (e) B. fallax (Cl. fallax)
>
> (f) B. sporogenes (Cl. sporogenes): nonpathogenic

6. These include the principal members of the group but other doubtfully or nonpathogenic species may be present. The identification and differentiation of the bacilli of this group is ordinarily quite difficult although the two principal members, B. welchii and B. oedematis maligni, are readily identified.

7. Wounds, including gangrene, may be also infected with B. tetani and it is good practice to include examination for this bacillus as described below.

Method.—1. It is advisable to collect some of the serous or serogaseous exudate or pus for the preparation of smears and cultures; the material may be collected on sterile swabs. Excised portions of necrotic tissue and spicules of bone are also suitable.

2. Prepare smears on slides and stain by the method of Gram. The above mentioned organisms occur as large, Gram-positive bacilli with or without capsules; spores are not present but occur in cultures or alkaline sugar-free medium. Staphylococci, streptococci and other Gram-positive and negative organisms mentioned above under Bacteriological Examination of Infected Wounds may be present.

3. Prepare cultures as follows: (a) a blood agar plate for aerobic cultivation: (b) a blood agar plate for anaerobic cultivation; (c) a tube of glucose hormone

2. Prepare thin smears on slides and stain by Gram; any of the following organisms may be present:

Gram-positive
- *Staphylococcus aureus* and *albus*
- Hemolytic and non hemolytic streptococci
- Pneumococcus (*Diplococcus pneumoniae*)
- B. diphtheriae (*Corynebacterium diphtheriae*)
- B. pseudodiphtheriae (*Corynebacterium pseudodiphtheriae*)
- B. Subtilis
- Spirochaeta vincentii

Gram-negative
- B. pyocyaneus (*Pseudomonas aeruginosa*)
- B. coli (*Escherichia coli*)
- B. proteus-vulgaris (especially in gun shot wounds)
- B. fusiformis (*Fusiformis dentium*)

3. Prepare cultures on blood agar plates (surface streak method) and in glucose hormone broth.

4. Incubate 24 to 48 hours. Prepare smears of the broth culture and of different colonies; stain by method of Gram. Employ special methods for identification of organisms.

Bacteriological Control of Treatment of Infected Wounds.—1. There should be no disinfectant treatment of the wound for at least two hours before smears and cultures are made.

2. Every two or three days smears should be made on slides with a sterile inoculating wire or sterile swabs, care being taken to obtain material from the worst parts of the wound.

3. Dry in the air. Fix with heat. Stain with methylene blue or by Gram's method.

4. Wash with water, dry and examine (with oil-immersion lens and No. 10 ocular).

5. Estimate the average number of bacteria per field. The results may be charted.

6. As long as there are over 50 bacteria per field, closure of the wound is contra-indicated.

7. If 50 or less, make the examination each day and likewise cultures on blood agar for hemolytic streptococci.

8. If smears show gram-positive bacilli resembling the anaerobes, make anaerobic cultures.

9. The presence of hemolytic streptococci in any number contraindicates closure.

10. The presence of a few saprophytes (2 or 3 per field) does not contra-indicate secondary suturing of the wound.

11. Cultures made from wounds recently treated with a chlorine compound may be freed from the antiseptic chlorine by obtaining the material on a sterile swab, immersing this in a tube of sterile N/10 sodium thiosulphate solution, and then preparing cultures from this neutralized mixture.

BACTERIOLOGICAL EXAMINATION OF GANGRENE OF WOUNDS AND METHODS FOR THE IDENTIFICATION OF PATHOGENIC AND NONPATHOGENIC ANAEROBIC BACILLI

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> (c) *B. oedematiens* (*Cl. oedematiens*) in "phlegmonous" gangrene
>
> (d) *B. histolyticus* (*Cl. histolyticus*)
>
> (e) *B. fallax* (*Cl. fallax*)
>
> (f) *B. sporogenes* (*Cl. sporogenes*); *nonpathogenic*

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3. Prepare cultures as follows: (a) a blood agar plate for aerobic cultivat (b) a blood agar plate for anaerobic cultivation; (c) a tube of glucose horn

broth for aerobic cultivation and (d) a deep tube of alkaline, sugar-free, cooked meat infusion broth for anaerobic cultivation. (e) Also boil a tube of glucose agar and cool to 42° C.; inoculate thoroughly; harden rapidly and cover with sterile paraffin oil or molten agar.

4. Incubate 24 to 48 hours and examine; prepare smears of colonies and stain by method of Gram.

5. Transplant colonies to alkaline, sugar-free infusion broth and cultivate anaerobically for 4 to 48 hours.

6. Prepare smears and stain by Gram method; test for motility. Subculture in litmus milk, cooked meat medium, gelatin, Löffler's blood serum and the following sugar broths for acid and gas: dextrose, lactose and salicin.

7. Incubate anaerobically for 1 to 6 days and identify according to Table I.

TABLE I

Name	Motility	Litmus milk	Cooked meat medium	Lique-faction Gelatin	Digest Blood Serum	Fermentation Dextrose	Fermentation Lactose	Fermentation Salicin	Patho-genicity
B. welchii	−	Acid Gas Clot Rapid "Stormy"	Gas Pink Butyric odor No digestion	+	−	AG	AG	−	Guinea-pigs Mice Pigeons
B. oedematis-maligni	+	Acid Clot Some gas 1 to 6 days	Gas Pink Rancid No digestion	+	−	AG	AG	AG	Guinea-pigs Rabbits Mice Pigeons
B. oedematiens	+	Acid 1 to 6 days Clot	Gas Pink Later bleached No digestion	+	−	AG	−	−	Guinea-pigs Rabbits Mice Pigeons
B. histolyticus	+	Soft clot Peptonized	Some gas Digestion No putrid odor	+	+	A	−	−	Usually Nonpatho-genic
B. fallax	+	Acid Gas Clot 3-7 days	Gas Pink No digestion	−	−	AG	−	?	Guinea-pigs Mice
B. sporogenes	+	Alkaline Clot Peptoniza-tion	Gas Blackened Digested Putrid	+	+	AG	−	−	Nonpatho-genic

Additional methods for the identification of the pathogenic and commoner non-pathogenic sporulating anaerobes have been recently described by Spray employing the following:

(a) *Iron Milk* prepared by mixing fresh whole milk and placing in tubes to

which are added strips of No. 26 gage black stove-pipe iron, cut about 50×7 mm. Autoclave as usual, but reduce the pressure slowly when completed to avoid wetting or blowing of plugs. The medium is used for gas production, coagulation, digestion and blackening. Some strains do not blacken the medium but produce a slight smoky browning; in the following table these are recorded as negative (—).

TABLE II

"Tentative Key" after Spray for the Identification of the Sporulating Anaerobes

Organisms	Iron Milk				Lead acet.	Sucrose*	Salicin*	Glucose*	Lactose*	Gelatin	Vanillin Violet	Nitrite	Indol	Motility†
	Gas	Coag.	Diges.	Blackened										
Cl. welchii	+	+	—	—	+	+	—	+	+	+	—	+	—	—
Vibrion septique	+	+	—	—	—	—	+	+	+	+	—	+	—	+
Cl. oedematiens	±	—	—	—	+	—	—	+	—	+	—	—	—	+
Cl. tetani	—	—	—	—	—	—	—	~	—	+	?	—	+	+
Cl. histolyticum	±	+ or —	+	+	—	—	—	—	—	+	—	—	—	+
Cl. sordelli	±	+ or —	+	+	+	—	—	+	—	+	?	—	+	+
Cl. chauvoei	±	+ or —	—	—	+	+	—	+	+	+	~	+	—	+
Cl. fallax	±	+	—	—	+	+	+	+	+	—	—	+	—	+
Cl. botulinum(A.B)	±	+ or —	+	+	+	—	+	+	—	+	?	—	—	+
Cl. botulinum (C)	±	+ or —	—	—	—	—	—	+	—	+	—	+	—	+
Cl. aerofoetidum	+	+	—	—	+	—	+	+	+	+	—	+	—	+
Cl. multifermentans	+	+	—	—	—	+	+	+	+	—	—	+	—	+
Cl. sphenoides	±	+	—	—	+	+	+	+	+	—	?	+	+	+
Cl. tertium	±	+	—	—	±	+	+	+	+	—	—	+	—	+
Cl. sporogenes	±	+ or —	+	+	+	—	—	+	—	+	?	—	—	+
Cl.tetanomorphum	—	+ or —	—	—	—	—	—	+	—	—	?	—	+	+

* Titrate end reaction at end of 36 hours; acidity is +; presence and amount of gas not reliable; questionable.
† Must be made early upon perceptible turbidity.

(b) Difco *lead acetate semisolid agar* for hydrogen sulphide.

(c) *Sucrose, salicin, glucose* and *lactose agars*: 1 per cent in agar prepared of 10 grams of Difco neopeptone, 10 grams Difco tryptone, 2.5 grams agar flakes and 1000 c.c. distilled water; pH 7.3 to 7.4; andrade or other indicators added or omitted; tubed; autoclaved.

Keep one part without sugar as a control. It is used for the detection of indol (Ehrlich and vanillin violet) tests being made 3 to 7 days after inoculation following a determination of the pH. The *vanillin violet* test is conducted by adding

10 drops of 5 per cent vanillin in 95 per cent alcohol followed by 10 drops of concentrated hydrochloric acid. The orange indol reaction appears in degree according to species.

(d) *Iron-gelatin* prepared of 128 grams Difco nutrient gelatin, 1 gram dextrose and 1000 c.c. distilled water. Dissolve in boiler; adjust to pH 7.3 to 7.4; tube and add to each one strip of iron; autoclave.

Incubate cultures at 37° C. and test daily in ice water for 10 days; if negative, paraffin the stoppers and test up to 30 days.

(e) *Nitrate semisolid agar* prepared of 5 grams of Difco tryptone, 5 grams of Difco neopeptone, 2.5 grams agar flakes and 1000 c.c. distilled water. Boil; adjust pH 7.3 to 7.4 and add 1 gram potassium nitrate and 0.5 gram glucose. Tube and autoclave.

Test after 72 hours' incubation using 0.6 per ccm dimethyl-alpha-naphthylamine (Eastman), with sulfanilic acid, both in dilute acetic acid.[1]

Animal Inoculation for B. Welchii.—1. Inject a rabbit intravenously with 1 to 3 c.c. of a saline suspension of infected material.

Fig. 234.—CLOSTRIDIUM WELCHII

1, Smear from wound. 2, Smear from culture. 3, Culture tube showing gas formation in agar. (From Park, Williams and Krumwiede, *Pathogenic Microörganisms*, Lea and Febiger.)

2. Five minutes later kill the animal and place the body in an incubator at 37° C. for 12 to 18 hours.

3. *B. welchii* produces distention with gas and bubbles may be formed in heart, arteries and liver. Prepare smears and stain by Gram. Prepare shake cultures in molten glucose agar cooled to 42° C. and incubate for 24 to 48 hours.

4. Large, gram-positive bacilli which may be encapsulated with "stormy fermentation" of the glucose agar are presumptive evidence of the presence of *B. welchii* (Fig. 234). The method is not infallible, however, as the findings may be due to postmortem invasion by anaerobic organisms from the intestinal tract of the animal.

Animal Protection Test for Diagnosis of Gaseous and Phlegmonous Gangrene.—1. Prepare a saline extract of macerated gangrenous tissue or wound secretion.

[1] *J. Bact.*, 1936, 32:135.

2. Place 1 c.c. into each of five small sterile test tubes.

3. Add 1 c.c. of the following antisera:

    No. 1: Tetanus antitoxin

    No. 2: Anti-welchii serum (*B. welchii*)

    No. 3: Anti-malignant edema serum (*Vibrion septique*)

    No. 4: Anti-oedematiens serum (*B. oedematiens*)

    No. 5: Saline solution (control)

4. Place in incubator for thirty minutes.

5. Inject into 5 guinea-pigs (subcutaneously) respectively.

6. The control becomes sick in 6 to 12 hours and usually dies.

7. The one or ones protected by serum show no reactions and indicate the nature of the infection.

BACTERIOLOGICAL EXAMINATION FOR B. TETANI AND THE DIAGNOSIS OF TETANUS

Principles.—1. So few tetanus bacilli are ordinarily present in wounds producing tetanus that the bacteriological diagnosis is usually quite difficult.

2. Whenever possible material for bacteriological examination like pus, tissue scrapings and foreign substances should be obtained from the depths of the wound. These may be collected on sterile swabs or emulsified in a small amount of sterile saline solution and sent to the laboratory.

Method.—1. Prepare smears and stain with carbolfuchsin or the method of Gram. Examine for moderately gram-positive bacilli with terminal spores. This method of examination, however, is of very little practical value.

2. Inoculate the material in (*a*) cooked meat medium; (*b*) in glucose hormone broth and (*c*) on a blood agar plate. Incubate at 37° C. for 72 hours under strict anaerobic conditions. Prepare and stain smears. Look for moderately gram-positive bacilli with terminal spores or drumstick forms. (Fig. 235).

3. Pure cultures are rarely found. For isolation heat the broth cultures at 75° to 80° C. for 30 minutes in a water bath to kill nonsporing organisms and inoculate blood agar plates for anaerobic cultivation. Also inoculate the water of condensation of an agar slant and cultivate anaerobically in an upright position. Tetanus bacilli produce an effuse, tenacious proteus-like growth over the surface of the slant. Subcultures from the edge of this fern-like growth into the water of condensation of a fresh agar slant will usually yield a pure culture after several transfers.

4. Test for motility; *B. tetani* are motile.

5. Inoculate litmus milk, cooked meat medium, Löffler's blood serum and fermentation tubes of dextrose, lactose and salicin broth. Cultivate anaerobically. *B. tetani* produces no change in milk; slight gas with no blackening or digestion of the cooked meat medium; no digestion of serum and no acid or gas with the three sugars.

6. Final identification may require a guinea-pig inoculation test. Inject an 8 to 10 ounce animal subcutaneously with 0.5 c.c. of a ten-day broth culture or its filtrate;

inject a second pig with the same dose along with 500 units of tetanus antitoxin. In the presence of tetanus bacilli the first animal will usually succumb with symptoms of tetanus within 4 days while the second survives.

Animal Inoculation Test.—1. So few tetanus bacilli and spores may be present in foreign matter, tissue scrapings, etc., from a wound as to escape bacteriological detection.

2. It is always advisable, therefore, to use a portion of the material for inocula-
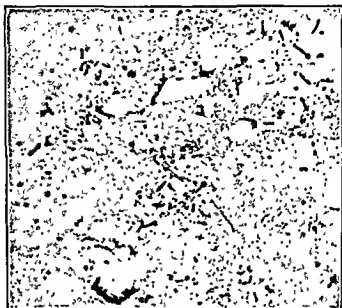
FIG. 235.—TETANUS BACILLI; SPORE STAIN (Zinsser)

tion of 3 to 10 ounce guinea-pigs. Prepare a finely divided emulsion in 2 or 3 c.c. of sterile saline. Inject 1 c.c. into the thigh of a pig. Inoculate a second pig with the same amount and also inject 500 units of tetanus antitoxin subcutaneously. In the presence of tetanus bacilli or spores the first animal is apt to develop tetanus and succumb in 1 to 4 days while the second survives with no symptoms.

3. In a clinical case of tetanus, toxin may be found in the cerebrospinal fluid. Inject 2 or 3 c.c. into the thigh of a young pig. Inject a second animal with the same amount along with 500 units of antitoxin. In the presence of toxin the first animal may develop symptoms or succumb in 1 to 4 days.

BACTERIOLOGICAL EXAMINATION OF LYMPHATIC GLANDS

Principles.—1. The bacteriology of the lymphatic glands is quite varied and their bacteriological examination is greatly aided by a knowledge of the clinical aspects of each case for the selection of technic to be employed.

2. Suppurative lymphadenitis is always likely to occur in association with infected wounds and lymphangitis. When pus is examined bacteriologically imme-

diately after spontaneous discharge or incision, the infecting organism is likely to be found in pure culture and is usually a *streptococcus* or *Staphylococcus aureus*.

3. Suppurative infection of the lymphatic glands of the neck is usually secondary to infection of the throat and the pus is likely to show a *streptococcus*, *Staphylococcus aureus*, a pneumococcus or a mixture of these organisms.

4. Any of the superficial lymphatic glands and especially those of the neck may become infected with *B. tuberculosis* producing tuberculous adenitis. When suppuration occurs infection with staphylococci or streptococci is commonly encountered.

5. Chronic open suppurative and tuberculous adenitis almost always shows mixed secondary infection or contamination with any of the following organisms in addition to those of the primary infection: *B. pyocyaneus*; *B. pseudodiphtheriae*; *B. coli*; *B. proteus-vulgaris*; *B. subtilis*, etc.

6. Suppurative adenitis of the groin in association with gonorrhea is usually due to mixed infection with the gonococcus, *Staphylococcus pyogenes* or a streptococcus.

7. In *chancroidal infection* the pus from the associated adenitis usually shows the *B. ducrey* in pure culture or in association with staphylococci and streptococci.

8. In bubonic plague *B. pestis* is the infecting organism.

9. Lymphadenitis is also commonly associated with the ulceroglandular type of *tularemia*. Bacteriological examination of the pus may show the presence of *B. tularense*, usually in association with a staphylococcus or a streptococcus; sometimes only either or both of the latter are found in smears and cultures.

10. The lymphatic glands are usually infected during the early stages of syphilis but rarely suppurate. Examination for *Spirochaeta pallida* is sometimes requested.

11. Suppurative lymphadenitis due to *mycotic infections* as in actinomycosis, sporotrichosis and blastomycosis is likely to show the primary organism in the pus usually in association with a staphylococcus, streptococcus or other organisms of secondary infection.

12. Chronic adenitis in association with Hodgkin's disease, lymphatic leukemia, etc., rarely suppurate and bacteriological examination of these, as well as the enlarged glands of cancer and sarcoma, are apt to reveal the presence of pseudodiphtheria bacilli and other organisms without etiological relationship to the primary disease.

Method for Examination of Pus in Suppurative Lymphadenitis.—1. The pus should be secured by incision or aspiration or as soon as possible after spontaneous rupture. It may be collected on sterile gauze or swabs.

2. Prepare thin smears on slides and stain by the method of Gram.

3. Inoculate a tube of glucose hormone broth and blood agar plates.

4. Incubate at 37° C. for 24 to 48 hours. Examine colonies; prepare smears and stain by Gram.

5. In ordinary acute lymphadenitis a *streptococcus* or *Staphylococcus aureus* are usually found. In chronic suppurative lymphadenitis these may be present along with organisms of secondary infection or contamination like *B. pyocyaneus*, *B. pseudodiphtheriae*, *B. proteus-vulgaris*, *B. coli*, *B. subtilis*, etc.

Method for Examination for Tubercle Bacilli.—1. Prepare smears on slides and stain by Ziehl-Neelson method.

2. Examine carefully for acid-fast bacilli.

3. If present they are likely to be tubercle bacilli but this diagnosis should be confirmed by culture and guinea-pig inoculation.

4. If a large amount of pus is available and direct smears are negative, one of the concentration methods described on page 482 may be employed.

5. Prepare cultures on Corper's crystal violet potato or Petroff medium as described on page 483.

6. Conduct a guinea-pig inoculation test as described on page 481.

7. If a gland has been removed, place a portion of 4% formalin for microscopical examination of sections. The balance should be finely minced or emulsified under aseptic conditions in sterile saline. Cultures are then prepared and guinea-pigs inoculated.

BACTERIOLOGICAL EXAMINATION OF FISTULAE

Principles.—1. Fistulae are usually chronic with mixed infection or contamination by two or more organisms.

2. They may occur in connection with tuberculous or pyogenic osteomyelitis and arthritis; following operations; in actinomycosis or other fungus infections of the skin; sarcoma and carcinoma, etc.

3. It is sometimes difficult to determine the nature of the primary infection because of the presence of secondary organisms like *B. pyocyaneus*, *B. pseudo-diphtheriae*, *B. proteus-vulgaris*, *B. coli*, *B. subtilis*, etc.

4. Whenever possible fragments of tissue or bone or scrapings should be examined rather than free pus. Otherwise material should be obtained from the deeper parts on sterile swabs.

Method.—1. Prepare thin smears on slides and stain by the Gram method. If tuberculosis is suspected stain by the Ziehl-Neelson method and examine carefully for acid-fast bacilli. If actinomycosis, blastomycosis or sporotrichosis are suspected examine wet preparations.

2. Prepare cultures on blood agar plates for the ordinary organisms. Bits of tissue or fragments of bone may be placed in glucose hormone broth, incubated at 37° C. for 24 hours, smears examined and plates prepared for isolation if more than one organism is present.

3. If tuberculosis is suspected, use Corper's or Petroff's medium although it is difficult to secure the tubercle bacilli under these conditions. Conduct a guinea-pig inoculation test.

4. If a pathogenic fungus or yeast is suspected use Sabouraud's medium.

BACTERIOLOGICAL EXAMINATION OF ULCERS

Principles.—1. Ulcers may occur on the skin, cornea and various mucous membranes (conjunctivae, nose, throat, mouth, vagina, urethra, bladder, rectum, etc.) due to a wide variety of infections with pathogenic bacteria, fungi, yeasts, etc.

2. The methods of examination to be employed, therefore, will largely depend upon the nature of the infection suspected.

3. Secondary infection or contamination is common and the bacteriological detection of the primary or specific infection may be difficult.

4. Ulcers due to vascular occlusion or trophic changes invariably become infected or contaminated with pathogenic and nonpathogenic organisms.

5. Material may be collected as described on page 424. The method is important, as mere surface collections may fail to reveal the organism of primary infection. An effort should be made to collect material from the base or depths of the ulcer after cleansing. Sometimes removal of tissue by biopsy is required for acceptable bacteriological examination.

Method.—1. Prepare thin smears. Stain by method of Gram. Stain for acid-fast bacilli if tuberculosis is suspected. Examine fresh material by dark-field method if syphilis or Vincent's infection is suspected. Examine wet preparations if infection with fungi or yeasts is suspected. Ordinarily such direct methods are more valuable than cultures for diagnostic purposes.

2. Inoculate glucose hormone broth and prepare blood agar plates by surface streak method for ordinary pyogenic organisms. Use Corper's or Petroff's media if infection with *B. tuberculosis* is suspected. Use Sabouraud's medium for fungi and yeasts.

3. Guinea-pig inoculation is best for the detection of *B. tuberculosis;* testicular inoculation of rabbits may be employed in the detection of *Spirochaeta pallida.*

4. Employ special methods for identification of organisms.

BACTERIOLOGICAL EXAMINATION OF THE EYE

Principles.—1. Infections of the eye in relation to bacteriological diagnosis are usually those involving the lids (hordeola and blepharitis); the conjunctivae (acute and chronic conjunctivitis); the cornea and fluid of the anterior chamber (acute and chronic keratitis with or without ulcers): the iris and anterior chamber fluid (in acute and chronic iritis); the lacrimal ducts and sac (dacrocystitis) and sometimes the lens, choroid, retina and fluid of the posterior chamber.

2. Smears and cultures of the conjunctival sac are also sometimes required before iridectomy and other operations for the purpose of determining whether or not preparatory disinfection is required.

3. Blood agar slants and plates as well as tubes of glucose hormone broth are suitable for the preparation of cultures. When pus and secretions are collected on sterile swabs, smears and cultures should be promptly made.

4. Material may be collected as described on page 317. Well prepared smears of pus or secretions are of great value in the bacteriological diagnosis of conjunctivitis. Local treatment should be stopped until sufficient is obtained for smears and cultures.

Method.—1. Stain thin smears by the method of Gram and examine. Additional smears may be stained with methylene blue or diluted carbolfuchsin. Fresh wet preparations should be made and examined in suspected infection with fungi and yeasts.

2. Cultures in glucose hormone broth, on blood agar plates or tubes should be incubated at 37° C. for 24 to 48 hours and examined. Special culture media are required for *B. tuberculosis*, *B. tularense*, fungi and yeasts, etc.

3. Material to be examined for *B. tuberculosis* may be examined in smears stained by the Ziehl-Neelson method but guinea-pig inoculation is required.

4. The following organisms may be found in infections of these parts:

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci, both hemolytic and nonhemolytic
- Pneumococci
- *B. diphtheriae* (*Corynebacterium diphtheriae*)
- *B. xerosis* (*Corynebacterium xerosis*)
- *B. anthracis*
- *B. subtilis*
- *Oidium albicans*
- Leptothrices
- Streptothrices
- *Actinomyces hominis*
- *Aspergillus fumigatus*

Gram-negative
- Gonococcus (*Neisseria gonorrhoeae*)
- *M. catarrhalis* (*Neisseria catarrhalis*)
- Meningococcus (*Neisseria intracellularis*)
- Bacillus Morax-Axenfeld (*Hemophilus lacunatus*)
- Bacillus Koch-Weeks (*Hemophilus conjunctivitidis*)
- *B. influenzae* (*Hemophilus influenzae*)
- *B. pyocyaneus* (*Pseudomonas aeruginosa*)
- *B. tularense* (*Pasteurella tularensis*)
- *B. Zur-Nedden*

BACTERIOLOGICAL EXAMINATION OF THE NOSE AND ACCESSORY SINUSES

Principles.—1. Methods for the collection of material are important and are described on page 318; as a general rule, these should be made by a rhinologist.

2. The bacteriology of these parts is quite varied and special methods are required for certain organisms.

3. In suspected leprosy prepare smears on slides and stain for acid-fast bacilli by the Ziehl-Neelson method. In leprosy of the skin a nodule may be removed by biopsy. Place a portion of 4% formalin for microscopical examination of sections. Smears may be prepared and stained for *B. leprae*.

4. Furuncles of the atrium are caused by *Staphylococcus aureus* or *albus*.

5. The etiological agent of the acute infectious "cold" or coryza is apparently the filtrable virus of Dochez. Later in the disease secondary infection with pyogenic and other organisms commonly occurs.

Method.—1. Prepare smears on slides and stain by method of Gram and

methylene blue. Smears for *Spirochaeta vincentii* and *B. fusiformis* should be stained with carbolfuchsin. Smears for tubercle bacilli should be stained by the Ziehl-Neelson method and carefully examined. In suspected chancre, a dark-field examination for *Spirochaeta pallida* is required. It is useless to examine for this organism in chronic syphilitic lesions, as ulcerated gummata.

2. Inoculate tubes of glucose hormone broth and blood agar for pyogenic organisms. Use Löffler's blood serum for *B. diphtheriae*. Use blood agar plates if mixed infection is suspected. as in chronic sinusitis.

3. Incubate 24 to 48 hours. Examine smears stained by the Gram method and methylene blue. Any of the following organisms may be present:

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *B. segmentosus* (*Corynebacterium segmentosum*)
- *B. diphtheriae* (*Corynebacterium diphtheriae*)
- *B. pseudodiphtheriae* (*Corynebacterium pseudodiphtheriae*)
- *B. subtilis*

Gram-negative
- *M. catarrhalis* (*Neisseria catarrhalis*)
- Meningococcus (*Neisseria intracellularis*)
- *B. Mucosus ozaenae* (*Klebsiella ozaenae*)
- *B. rhinoscleroma* (*Klebsiella rhinoscleromatis*)
- Friedländer's bacillus (*Klebsiella pneumoniae*)
- *B. pyocyaneus* (*Pseudomonas aeruginosa*)
- *B. influenzae* (*Hemophilus influenzae*)
- *B. fusiformis* (*Fusiformis dentium*)
- *B. pertussis* (*Hemophilus pertussis*)
- *B. mallei* (*Pfeifferella mallei*)
- *B. proteus vulgaris*
- *Spirochaeta vincentii* (*Borrelia vincentii*)

Acid-fast
- *B. tuberculosis* (*Mycobacterium tuberculosis*)
- *B. leprae* (*Mycobacterium leprae*)

BACTERIOLOGICAL EXAMINATION OF THE THROAT INCLUDING NASOPHARYNX AND TONSILS

Principles.—1. It is important that smears and cultures be properly prepared: methods are described on page 318.

2. Whenever possible the particular infection suspected or to be examined for should be stated to guide technic, choice of culture medium, etc.

3. Smears on slides or wet preparations for dark-field examination are required for the detection of *Spirochaeta vincentii* and *B. fusiformis*. Smears may be of some value in the detection of diphtheria bacilli, but when negative should never be allowed to exclude the possibility of this infection. With the exception of

Vincent's angina and tuberculosis, cultures are required for the bacteriological examination of these parts.

Method.—1. Smears on slides prepared direct or from swabs for examination for Vincent's infection may be stained with dilute carbolfuchsin; otherwise the method of Gram is preferred.

2. Smears for tubercle and leprae bacilli should be stained by the Ziehl-Neelson method.

3. For diphtheria bacilli use tubes or plates of Löffler's blood serum media. For the meningococcus, sheep serum or other special media in plates may be employed. For other organisms as streptococci, staphylococci, pneumococci and bacilli of the hemophilic group, inoculate plates of blood agar and tubes of glucose hormone broth.

4. Incubate 24 to 48 hours. Examine colonies. Prepare smears and stain by the Gram method; the following organisms constitute the more important of these parts:

Gram-positive
$$\begin{cases}\text{Staphylococcus aureus and albus}\\ \text{Streptococci}\\ \text{Pneumococci}\\ \text{B. diphtheriae (Corynebacterium diphtheriae)}\\ \text{B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)}\end{cases}$$

Gram-negative
$$\begin{cases}\text{M. catarrhalis (Neisseria catarrhalis)}\\ \text{M. flavus (Diplococcus perflava)}\\ \text{M. pharyngis siccus (Diplococcus siccus)}\\ \text{Meningococcus (Neisseria intracellularis)}\\ \text{B. influenzae (Hemophilus influenzae)}\\ \text{B. fusiformis (Fusiformis dentium)}\\ \text{B. pertussis (Hemophilus pertussis)}\\ \text{B. pyocyaneus (Pseudomonas aeruginosa)}\\ \text{B. proteus vulgaris}\\ \text{Friedländer's bacillus (Klebsiella pneumoniae)}\\ \text{Spirochaeta vincenti (Borrelia vincenti)}\end{cases}$$

Acid-fast
$$\begin{cases}\text{B. tuberculosis (Myobacterium tuberculosis)}\\ \text{B. leprae (Mycobacterium leprae)}\end{cases}$$

BACTERIOLOGY OF THE MOUTH INCLUDING THE TEETH AND GINGIVAE

Principles.—1. The collection of material for the bacteriological examination of the teeth and gingivae is very important and methods are described on page 320.

2. Gingival pus and secretions require the examination of fresh wet preparations for spirochetes and endamebae as well as the examination of stained smears and cultures.

3. The results of dark-field examination of ulcers for *Spirochaeta pallida* must be interpreted with great care and caution because of the chances of error with

Spirochaeta microdentium and other spirochetes commonly found in the saliva.

4. For mycotic infections the examination of fresh wet preparations and stained smears is required in addition to cultures.

Method.—1. Prepare smears and stain with diluted carbolfuchsin and by the method of Gram.

2. Prepare and examine wet preparations for *Endameba gingivalis, Oidium albicans.* etc.

3. Dark-field examination of fresh wet preparations may be conducted for the detection of spirochetes.

4. Prepare cultures on plates of blood agar by the surface streak method. The apices of extracted teeth are best cultured in Rosenow's brain broth or glucose hormone broth. After incubation for 24 to 48 hours prepare smears stained by Gram method. For the isolation of mixed cultures inoculate blood agar plates by the surface streak method. Anaerobic cultures are sometimes required.

5 The bacterial flora is extensive and variable; the chief organisms are as follows:

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *M. tetragenus (Gaffkya tetragena)*
- *B. diphtheriae (Corynebacterium diphtheriae)*
- *B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*
- *B. acidophilus (Lactobacillus acidophilus)*
- *B. mesentericus ruber (B. teres)*
- *B. mesentericus-vulgatus (B. graveolens)*
- *B. mesentericus-fuscus (B. mesentericus)*
- Various sarcinae
- *Leptotrichia buccalis*
- Streptothrices
- *Oidium albicans*

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- *B. fusiformis (Fusiformis dentium)*
- *B. coli (Escherichia coli)*
- *B. influenzae (Hemophilus influenzae)*
- *Vibrio sputigenus*
- *B. proteus—vulgaris (Proteus zenkeri)*
- *Spirochaeta vincentii (Borrelia vincentii)*
- *Spirochaeta microdentium (Treponema microdentium)*
- *Spirochaeta macrodentium (Treponema macrodentium)*
- *Spirochaeta mucosum (Treponema mucosum)*

Acid-fast
- *B. tuberculosis (Myobacterium tuberculosis)*

BACTERIOLOGICAL EXAMINATION OF THE EARS AND MASTOIDS

Principles.—1. It is always advisable to prepare cultures of pus in acute otitis media as soon as possible after paracentesis typani or after spontaneous rupture, since pure cultures of the infecting organism are thereby usually obtained. In chronic suppurative otitis media, mixed cultures are the rule.

2. It is good practice to routinely culture all cases of mastoiditis at the time of operation. Pure cultures are the rule and in case of subsequent complications, like lateral sinus thrombosis with septicemia or meningitis, valuable information will be previously obtained.

3. The technic of obtaining and culturing material of these parts for bacteriological examination is very important and described on page 322.

Method.—1. Stain smears by the method of Gram and examine. They are of limited value and cultures are preferred.

2. Cultures are best prepared on slants or plates (preferred) of blood agar; plain agar should not be used. Löffler's blood serum is acceptable. At the same time it is advisable to inoculate tubes of glucose hormone broth or Rosenow's brain broth medium as these are best for the cultivation of streptococci and pneumococci.

3. Incubate 24 to 48 hours. Examine colonies. Prepare and stain smears by Gram method. Streptococci must be differentiated from pneumococci. Identify organisms by proper methods:

Gram-positive
- *Staphylococcus aureus and albus*
- Streptococci
- Pneumococci
- B. *diphtheriae (Corynebacterium diphtheriae)*
- B. *pseudodiphtheriae (Corynebacterium pseudodiphtheriae)*

Gram-negative
- *M. catarrhalis (Neisseria catarrhalis)*
- Meningococcus (*Neisseria intracellularis*)
- B. *pyocyaneus (Pseudomonas aeruginosa)*
- B. *influenzae (Hemophilus influenzae)*
- B. *fusiformis (Fusiformis dentium)*
- Friedländer's bacillus (*Klebsiella pneumoniae*)
- B. *proteus vulgaris*
- *Spirochaeta vincenti (Borrelia vincenti)*

Acid-fast
- B. *tuberculosis (Mycobacterium tuberculosis)*

BACTERIOLOGY OF THE SPUTUM INCLUDING SECRETIONS AND PUS OBTAINED BY BRONCHOSCOPIC DRAINAGE

Principles.—1. Methods for the collection of sputum and bronchial secretions for bacteriological examination are given on page 319. They are particularly im-

portant in relation to bacteriological diagnosis and the preparation of autogenous vaccines.

2. In the different types of suppurative pneumonitis (lung abscess; bronchiectasis, etc.) anaerobic cultures should be included in addition to aerobic cultures.

3. The bacterial flora ordinarily includes many different bacteria as well as various fungi and yeasts. Whenever possible the particular organism suspected clinically should be specified in order to guide the bacteriological technic.

Method.—1. Routinely prepare and carefully examine smears stained by the Ziehl-Neelson technic for tubercle bacilli. Several specimens of sputum may be required for examination before tubercle bacilli are found.

2. Cultures for tubercle bacilli on Corper's, Petroff's or other special media are advisable.

3. Guinea-pig inoculation for tubercle bacilli may be required.

4. Make a dark-field examination for spirochetes or smears may be stained with diluted carbolfuchsin. Prepare smears and stain by the method of Gram.

5. Prepare and examine fresh wet smears for fungi and yeasts. Prepare cultures on Sabouraud's medium, if infection with these organisms is suspected.

6. Prepare blood agar plates by the surface streak method or inoculate tubes of glucose hormone or Rosenow's brain broth media and plate after 24 hours' incubation. It is advisable to include anaerobic incubation with duplicate cultures.

7. The more important organisms are as follows:

Gram-positive
- *Staphylococcus aureus* and *albus*
- Streptococci
- Pneumococci
- *M. tetragenus* (*Gaffkya tetragena*)
- *B. diphtheriae* (*Corynebacterium diphtheriae*)
- *B. pseudodiphtheriae* (*Corynebacterium pseudodiphtheriae*)
- Blastomycetes
- Actinomyces
- *Actinomyces hominis*
- *Actinomyces asteroides*
- Streptothrices
- *Leptotrichia buccalis*
- *Oidium albicans*

Gram-negative
- *M. catarrhalis* (*Neisseria catarrhalis*)
- Meningococcus (*Neisseria intracellularis*)
- Friedländer's bacillus (*Klebsiella pneumoniae*)
- *B. pyocyaneus* (*Pseudomonas aeruginosa*)
- *B. fusiformis* (*Fusiformis dentium*)

Gram-negative
{
B. pertussis (Hemophilus pertussis)
B. influenzae (Hemophilus influenzae)
B. proteus vulgaris
B. coli (Escherichia coli)
Spirochaeta vincenti (Borrelia vincenti)
Spirochaeta microdentium (Treponema microdentium)
Spirochaeta macrodentium (Treponema macrodentium)
Spirochaeta mucosum (Treponema mucosum)
}

Acid-fast { B. tuberculosis (Mycobacterium tuberculosis)

BACTERIOLOGICAL EXAMINATION OF THE CEREBROSPINAL FLUID

Principles.—1. Cerebrospinal fluid should be collected under rigid aseptic precautions to prevent accidental contamination with *Staphylococcus albus* and other organisms of the skin; a method is described on page 296.

2. Bacteriological examination should be made as soon as possible after collection as otherwise delicate organisms and especially the meningococcus, may not survive.

3. Relatively large amounts of fluid or sediment should be cultured (0.5 to 1 c.c.) instead of only 1 or 2 loopsful.

4. Smears and cultures are required routinely. Fluids showing no organisms in direct smears may yield positive cultures; or organisms may be found in direct smears of fluid or sediment with sterile cultures.

5. Spinal fluid is normally crystal clear. Opalescence to turbidity usually indicates the presence of pleocytosis due to meningitis in case blood is absent. Perfectly clear fluids, however, may be observed in tuberculous meningitis.

Method.—1. The examination of stained smears is very important. These may be prepared of cloudy fluid. It is better to remove a portion with a sterile pipet; centrifuge thoroughly. The supernatant fluid may be used for protein, sugar and other determinations. Prepare smears of the sediment. Stain by the method of Gram and with methylene blue. Definitely gram-negative diplococci (intracellular or extracellular) justifies a provisional diagnosis of meningococcus meningitis and the prompt administration of serum.

2. In suspected tuberculous meningitis stain smears by the Ziehl-Neelson method. If a coagulum has formed, carefully tease out a portion on a slide, dry and stain. A careful search should be made for acid-fast bacilli (usually requires about an hour).

3. With a sterile pipet inoculate 0.5 to 1 c.c. on a blood agar slant or plate. Do not use plain agar. It is advisable to inoculate a tube of glucose hormone broth at the same time with 0.5 to 1 c.c. Löffler's blood serum medium is also serviceable. Incubate 24 to 48 hours. Examine cultures. Prepare smears stained by the method of Gram. Identify organisms. Gram-negative diplococci are usually the meningococcus. Gram-negative bacilli are usually *B. influenzae*. Gram-positive cocci in chains are usually hemolytic streptococci or a pneumococcus. Differentiate

by appearance of colonies, bile solubility, inulin fermentation and agglutination tests.

4. For tubercle bacilli use Corper's or Petroff's media. Inoculate guinea-pig.

5. *Spirochaeta pallida* is best detected by intratesticular inoculation of rabbits with 1 to 3 c.c. of fluid immediately after removal.

6. Acute *primary* meningitis is usually due to the meningococcus, streptococcus, pneumococcus or *B. influenzae.*

7. Acute *secondary* meningitis complicating otitis media, mastoiditis, sinusitis and injury is usually due to the streptococcus, pneumococcus or staphylococcus. These and other organisms less commonly encountered are as follows:

Gram-positive
- Streptococci
- Pneumococci
- Staphylococci
- *M. tetragenus (Gaffkya tetragena)*
- Actinomyces
- Yeasts

Gram-negative
- Meningococcus *(Neisseria intracellularis)*
- *B. influenzae (Hemophilus influenzae)*
- *B. coli (Escherichia coli)*
- *B. typhosus (Eberthella typhi)*
- *B. pestis (Pasteurella pestis)*

Acid-fast
- *B. tuberculosis (Mycobacterium tuberculosis)*

BACTERIOLOGICAL EXAMINATION OF PERITONEAL EXUDATES AND TRANSUDATES

Principles.—1. Peritoneal transudates (*ascites*) when collected aseptically are usually sterile but sometimes contain diphtheroid bacilli or other organisms detected by aerobic or anaerobic cultivation. Fluids are sometimes contaminated with *Staphylococcus albus* and other organisms from the skin.

2. Peritoneal exudates (pus) in cases of localized or diffuse peritonitis may show one or more organisms depending upon the cause of peritonitis. In suppurative appendicitis with rupture and in peritonitis following the rupture of peptic ulcers and perforating wounds of the intestines, the infection is always a mixed one with intestinal organisms and anaerobic cultures for *B. welchii* and other anaerobic spore-forming bacilli should be included in the bacteriological examination.

Method.—1. Centrifuge and prepare smears of the sediment; stain by the method of Gram.

2. Inoculate blood agar plates with 0.5 to 1 c.c.: also inoculate tubes of Rosenow's brain broth or glucose hormone broth. Cultivate one set aerobically for 24

to 48 hours and the second set anaerobically for several days. Examine colonies. Prepare smears and stain by the method of Gram. Identify organisms.

3. If tuberculous peritonitis is suspected stain smears by the Ziehl-Neelson method and examine very carefully for acid-fast bacilli. Prepare cultures on Corper's or Petroff's media; inoculate guinea-pigs.

4. The bacteriology may be quite varied but the following embrace the organisms usually encountered:

Gram-positive
$\begin{cases} \text{Staphylococci} \\ \text{Streptococci} \\ \text{Pneumococci} \\ \textit{B. welchii (B. aerogenes-capsulatus)} \\ \textit{B. pseudodiphtheria (Corynebacterium pseudodiphtheriae)} \\ \textit{B. subtilis} \end{cases}$

Gram-negative
$\begin{cases} \textit{B. coli (Escherichia coli)} \\ \textit{B. pyocyaneus (Pseudomonas aeruginosa)} \\ \textit{B. proteus—vulgaris} \\ \textit{B. typhosus (Eberthella typhi)} \end{cases}$

Acid-fast
$\begin{cases} \textit{B. tuberculosis (Mycobacterium tuberculosis)} \end{cases}$

BACTERIOLOGICAL EXAMINATION OF PLEURAL AND PERICARDIAL EXUDATES AND TRANSUDATES

Principles.—1. Pleural and pericardial transudates are usually sterile when collected aseptically. They are sometimes found contaminated with *Staphylococcus albus* or other organisms from the skin.

2. In pleuritis (empyema) the pus collected by aspiration usually shows infection due to pneumococci, hemolytic streptococci, *Staphylococcus aureus* or *B. influenzae*. In chronic pleuritis with drainage organisms of secondary infection or contamination are not unusual as described under the Bacteriological Examination of Fistulae.

3. In pericarditis the exudate or pus usually shows the presence of a streptococcus, pneumococcus or staphylococcus. In pericarditis following trauma additional organisms may be found.

Method.—1. Centrifuge if necessary and prepare smears of the sediment. Stain by the method of Gram. If tuberculosis is suspected stain by the method of Ziehl-Neelson and examine very carefully for acid-fast bacilli.

2. Inoculate plates of blood agar by the surface streak method. Also inoculate tubes of Rosenow's brain broth or glucose hormone broth and incubate at 37° C. for 24 to 48 hours. If tuberculosis is suspected inoculate tubes of Corper's or Petroff's media; inoculate guinea pigs.

3. Examine colonies. Prepare and stain smears by the method of Gram. Identify organisms present. The following include those usually present:

Gram-positive $\begin{cases}\text{Staphylococci}\\ \text{Streptococci}\\ \text{Pneumococci}\\ \textit{B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)}\end{cases}$

Gram-negative $\begin{cases}\textit{M. catarrhalis (Neisseria catarrhalis)}\\ \text{Friedlander's bacillus } \textit{(Klebsiella pneumoniae)}\\ \textit{B. influenzae (Hemophilus influenzae)}\\ \textit{B. pyocyaneus (Pseudomonas aeruginosa)}\\ \textit{B. proteus—vulgaris}\end{cases}$

Acid-fast $\begin{cases}\textit{B. tuberculosis (Mycobacterium tuberculosis)}\end{cases}$

BACTERIOLOGICAL EXAMINATION OF BILE AND GALLSTONES

Principles.—1. Bile is best obtained for bacteriological examination by aspiration of the gallbladder at operation or following the surgical removal of the gallbladder.

2. Bile collected by the Lyon method of duodenal drainage is subject to contamination by saliva, by the stomach and duodenum but when collected with rigid attention to technic as described in Chapter IX is usually acceptable for bacteriological examination.

3. At the time of drainage about 20 drops should be added to 150 c.c. of hormone broth with a pH of 7.4 to 7.6 suitable for the cultivation of streptococci.

4. Fractions may be collected on sterile vials or test tubes.

Method.—1. Centrifuge a portion; prepare smears and stain by method of Gram. The direct examination of wet preparations is also recommended.

2. With a sterile pipet inoculate 0.5 to 1 c.c. on blood agar plates by the surface streak method.

3. If a culture in hormone broth has not been made at the time of operation or drainage, inoculate a flask with 0.5 to 1 c.c.

4. A duplicate set of cultures for anaerobic cultivation is advisable.

5. Incubate at 37° C. for 24 to 72 hours. Examine colonies. Prepare smears and stain by Gram method. Identify organisms. The following include those generally found in pure or mixed culture:

Gram-positive $\begin{cases}\text{Staphylococci}\\ \text{Streptococci}\\ \textit{M. tetragenus (Gaffkya tetragena)}\\ \textit{B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)}\\ \textit{B. subtilis}\\ \textit{Saccharomyces cerevisiae, } \text{etc.}\end{cases}$

$$\text{Gram-negative} \begin{cases} \textit{B. coli (Escherichia coli)} \\ \textit{B. typhosus (Eberthella typhi)} \\ \textit{B. pyocyaneus (Pseudomonas aeruginosa)} \\ \textit{Friedländer's bacillus (Klebsiella pneumoniae)} \\ \textit{B. proteus—vulgaris} \end{cases}$$

6. Gallstones may be dipped for a few seconds in boiling water for surface sterilization. Crush in a mortar under rigid aseptic precautions and culture in a flask of plain or glucose hormone broth. Incubate at 37° C. for 24 to 48 hours. If a growth occurs, prepare smears stained by method of Gram. If a mixed growth is present, plate on blood agar by the surface streak method. Incubate at 37° C. for 24 to 48 hours. Examine colonies; prepare smears and stain by Gram method. Identify organisms. Those usually present are included among the organisms listed above.

BACTERIOLOGICAL EXAMINATION OF THE FECES AND RECTUM

Principles.—1. The feces contain so many different varieties of bacteria that a general bacteriological examination is inadvisable.

2. All requests, therefore, should specify the particular organism or organisms to be examined for in order to choose the proper culture media and technic.

3. The method of collection of material for bacteriological examination is important and is described on page 323. In the bacteriological examination of ulcers of the colon and rectum the material should be collected by a proctologist as described.

4. Mucus and pus are especially desirable for bacteriological examination.

Method.—1. Prepare thin smears and stain by the method of Gram. Their only value is an estimated percentage of gram-positive and gram-negative organisms. None of the pathogenic organisms can be identified by smear alone except in the case of tubercle bacilli detected in smears stained by the Ziehl-Neelson method.

2. For the detection of bacilli of the typhoid-paratyphoid-dysentery and cholera groups see special methods under the respective organisms.

3. For general bacteriological examination as for streptococci, staphylococci, etc., a loopful of material may be diluted in a tube of broth or sterile saline solution; mix well and transfer two or three loopsful to a second tube. Prepare blood agar plates from each dilution by the surface streak method. Or a loopful of material may be grown in a tube of Rosenow's brain or hormone broth for 24 hours and used for the preparation of blood agar plates. Incubate at 37° C. for 24 to 48 hours. Study the colonies; prepare smears and stain by the Gram method. Identify the different organisms.

4. For *B. welchii, B. tetani* and other pathogenic anaerobic spore forming bacilli use strict anaerobic cultivation for several days.

5. If acid-fast bacilli are found in direct smears or after concentration, inoculate guinea-pigs.

6. The organisms of most interest are as follows:

Gram-positive
- Staphylococci
- Streptococci
- Pneumococci
- B. welchii ⎫
- B. tetani ⎬ and other anaerobes
- B. acuminatus (Bacteroides acuminatus)
- B. pseudodiphtheriae (Corynebacterium pseudodiphtheriae)
- B. subtilis
- B. acidophilus and lactic acid groups
- B. bifudus (Bacteroides bifudus)
- Monilia psilosis

Gram-negative
- Gonococcus (Neisseria gonorrhoeae)
- B. coli (Escherichia coli)
- B. cholerae (Vibrio comma)
- B. typhosus (Eberthella typhi)
- B. paratyphosus A (Salmonella paratyphi)
- B. paratyphosus B (Salmonella schottmuelleri)
- B. enteritidis (Salmonella enteritidis)
- B. dysenteriae (Shigella dysenteriae)
- B. faecalis-alcaligenes
- B. ambiguus (Shigella ambigua)
- B. liquefaciens (Bacteroides liquefaciens)
- B. proteus vulgaris

Acid-fast
- B. tuberculosis (Mycobacterium tuberculosis)

BACTERIOLOGICAL EXAMINATION OF THE URINE

Principles.—1. Urine for bacteriological examination should be collected with precautions against contamination. Methods are described on page 326.

2. It is always advisable to state the particular organism or organisms to be examined for in order to properly select special methods for examination.

3. Cultures should be made as soon as possible after collection.

4. Urinary antiseptics should not be administered for 24 to 48 hours previous to the collection of the specimen.

5. A method for examination for tubercle bacilli is given on page 480; for typhoid-paratyphoid bacilli on page 489; for gonococci on page 454. A general examination for other organisms may be conducted as follows:

Method.—1. With a sterile pipet transfer 1 c.c. of urine to a tube of hormone broth and 0.5 c.c. to a plate of blood agar.

2. Centrifuge 5 to 10 c.c. under rigid aseptic precautions. Remove the supernatant urine and prepare duplicate cultures with smaller amounts of sediment.

3. Prepare thin smears of the sediment and stain by method of Gram, and by the Pappenheim method for acid-fast bacilli.

4. Examine cultures after incubation at 37° C. for 21 to 48 hours. Prepare smears and stain by Gram. Identify organisms if any are present.

5. Normally urine is sterile when aseptically collected, but under pathological conditions any of the following may be present:

Gram-positive
$\begin{cases} \text{Staphylococci} \\ \text{Streptococci} \\ \text{B. pseudodiphtheriae (Corynebacterium pseudodiphtherine)} \end{cases}$

Gram-negative
$\begin{cases} \text{Gonococcus (Neisseria gonorrhaeae)} \\ \text{M. catarrhalis (Neisseria catarrhalis)} \\ \text{B. proteus—vulgaris} \\ \text{B. pyocyaneus (Pseudamanas aeruginosa)} \\ \text{B. typhosus (Eberthella typhi)} \\ \text{B. paratyphosus A (Salmonella paratyphi)} \\ \text{B. paratyphosus B (Salmonella schottmuelleri)} \\ \text{B. coli (Escherichia coli)} \\ \text{B. dysenteriae (Shigella dysenteriae)} \\ \text{B. abortus (Brucella abortus)} \end{cases}$

Acid-fast
$\begin{cases} \text{B. tuberculosis (Mycobacterium tuberculosis)} \\ \text{B. smegmatis (Mycobacterium smegmatis)} \end{cases}$

BACTERIOLOGICAL EXAMINATION OF THE UROGENITAL ORGANS INCLUDING THE PROSTATE GLAND

Principles.—1. *Spirochaeta pallida* in genital sores is best detected by dark-field examination. Methods for the collection of material are given on page 328.

2. Examinations for *gonococci* are best conducted with smears of pus stained by the Gram method sometimes supplemented by cultures. Smears and cultures of urine sediment are sometimes employed but are not usually satisfactory. Vaginal washings are useful and a method for collection is given on page 327.

3. A method for the collection of material for examination for *B. ducrey* (chancroid) is given on page 330.

4. Material from the prostate gland for bacteriological examination may be obtained by methods given on page 327.

5. Urine collected by catherization from the bladder or kidneys may be examined for tubercle bacilli as described on page 480; for general examination the method previously given may be employed.

6. The organisms most commonly found in infections of the urogenital organs may be listed as follows:

Gram-positive
$\begin{cases} \text{Staphylococci} \\ \text{Streptococci} \\ \text{Pneumococci} \\ \text{B. diphtheriae (Carynebacterium diphtheriae)} \\ \text{B. pseudodiphtheriae (Carynebacterium pseudodiphtheriae)} \end{cases}$

Gram-negative
{
Gonococcus (*Neisseria gonorrhoeae*)
B. *proteus—vulgaris*
Bacillus of Ducrey (*Hemophilus ducreii*)
B. *coli* (*Escherichia coli*)
B. *acidophilus* (*Lactobacillus acidophilus*)
Spirochaeta refringens (*Borrelia refringens*)
}

Acid-fast
{
B. *tuberculosis* (*Mycobacterium tuberculosis*)
B. *smegmalis* (*Mycobacterium smegmalis*)
}

BACTERIOLOGICAL EXAMINATION OF THE BLOOD

Principles.—1. Bacteria are but rarely found in direct smears of the blood. Cultures are required and the technic is very important; methods are given on page 324.

2. Anaerobic cultures are sometimes advisable and especially in suspected streptococcus infections.

3. Bacteremia may be present without demonstrable clinical manifestations, but the number of organisms present is apt to be small, requiring the cultivation of relatively large amounts of blood.

4. The complement and natural antibodies of the blood may inhibit the growth of small numbers of organisms. For this reason the blood should be sufficiently diluted with culture medium. No culture should be finally reported as sterile unless incubation has been conducted for at least 10 days and in some instances for 14 to 21 days.

5. Postmortem blood cultures of the heart should be made within an hour after death, before general bacterial invasion of the tissues has occurred.

6. Organisms producing large amounts of exogenous toxins like the diphtheria and tetanus bacilli rarely produce septicemia. B. *welchii* is sometimes found but usually just before death or postmortem.

7. *Spirochaeta pallida* may be found during the acute early stages of syphilis but is only detected by the intratesticular inoculation of rabbits. *Spirochaeta recurrentis* may be detected by dark-field examination of the blood or by the examination of stained smears.

8. Staphylococci, diphtheroid bacilli, B. *subtilis*, B. *coli* and other organisms from the skin may contaminate blood cultures. When any of these are found, the culture should be repeated.

9. The bacteria most commonly encountered in blood cultures are as follows:

Gram-positive
{
Staphylococci
Streptococci
Pneumococci
B. *anthracis*
B. *welchii*, etc.
}

Meningococcus (*Neisseria intracellularis*)

Gram-negative
{
Gonococcus (*Neisseria gonorrhoeae*)
B. abortus (*Brucella abortus*)
B. typhosus (*Eberthella typhi*)
B. paratyphosus A (*Solmonella paratyphi*)
B. paratyphosus B (*Salmonella schottmuelleri*)
B. dysenteriae (*Shigella dysenterioe*)
B. coli (*Esherichia coli*)
B. pyocyoneus (*Pseudomonas oeruginoso*)
Friedländer's bacillus (*Klebsiello pneumoniae*)
B. influenzae (*Hemophilus influenzae*)
B. proteus vulgaris
B. pestis (*Posteurello pestis*)
Spiroehaeta obermeirei (*Borrelia recurrentis*)
Spirochaeta novyi (*Borrelia novyi*)
}

Acid-fast   B. tuberculosis (*Mycobacterium tuberculosis*)

BACTERIOLOGICAL EXAMINATION OF THE SKIN

Principles.—1. Many different kinds of organisms may be found on the skin in health and disease.

2. Requests should specify the kind of examination desired to determine the proper selection of methods, culture media, etc.

3. The bacteria, fungi and yeasts most commonly encountered are as follows:

Gram-positive
{
Staphylococci
Streptococci including *Streptococcus scarlatinae* and *Streptococcus erysipelatis*
Pneumococci
B. diphtheriae (*Corynebacterium diphtherine*)
B. pseudodiphtheriae (*Corynebacterium pseudodiphtheriae*)
B. acnes (*Corynebacterium acnes*)
B. onthracis
Erysipelothrix (*Erysipelothrix rhusiopathiae*)
Actinomyces { *Actinomyces hominis* / *Actinomyces maduroe* }
Sporotrichia (*Sporotrichum beurmanni*)
Blastomycetes
Tineo trichophytino {
Microsporon audouini
Trochophyton
Achorion schoenleinii
Microsporon furfur
Microsporon minutissimum
}
}

Gram-negative
{
B. pyocyaneus (*Pseudomonas aeruginosa*)
B. mallei (*Pfeifferella mallei*)
B. coli (*Escherichia coli*)
B. catarrhalis (*Neisseria catarrhalis*)
B. proteus vulgaris
Spirochaeta vincentii
}

Acid-fast
{
B. tuberculosis (*Mycobacterium tuberculosis*)
B. leprae (*Mycobacterium leprae*)
B. smegmatis (*Mycobacterium smegmatis*)
}

4. All local treatment and especially with germicidal agents should be omitted for at least 24 hours before bacteriological examinations are made.

5. A method for the collection of material for examination for *B. leprae* is given on page 330.

6. For other organisms including the yeasts, molds and fungi, scrapings with a sterile scalpel are desirable. Mere surface swabbings are of little or no value. Pieces of skin aseptically removed by biopsy are sometimes required.

METHODS FOR THE IDENTIFICATION OF STAPHYLOCOCCI

1. Staphylococci may occur singly, in pairs or in very short irregular chains but usually in grape-like clusters (Fig. 236), rarely in packets.

2. They stain readily and are gram-positive but in both smears of pus and cultures gram-negative cocci may occur. They are not encapsulated.

3. They grow readily in all ordinary culture media. On solid media the colonies are circular and opaque with smooth glistening surfaces and even edges. On blood agar many strains and especially *Staphylococcus aureus* produce zones of hemolysis. In broth they produce uniform turbidity with moderate deposits readily disintegrating on shaking. Gelatin is usually liquefied in five days at 22° C. and milk coagulated.

Fig. 236.—Staphylococcus Pyogenes Aureus.
(After Günther)

4. After two or three days of aerobic cultivation on solid media pigment may be produced in the colonies which is of great value in identifying the type:

   (a) Golden-orange: *Staphylococcus aureus*
   (b) Lemon-yellow: *Staphylococcus citreus*

(c) No pigment (white): *Staphylococcus albus*

5. Identification and differentiation can usually be made by these means. Always report the type present and whether hemolytic or nonhemolytic.

6. *Staphylococcus epidermidis albus* is merely a special type found on the skin and in "stitch abscesses"; it ferments sucrose but not mannitol and raffinose, whereas *Staphylococcus albus* ferments sucrose and mannitol but not raffinose.

7. *Staphylococcus aureus* usually produces several filtrable toxins now commonly converted into toxoids for purposes of active immunization. These are secured by cultivation in hormone broth (pH 7.4-7.6) for 5 days, followed by Berkefeld filtration. The filtrate may contain the following: (1) hemolysin especially active for rabbit erythrocytes; (2) leukocidin; (3) a necrotizing toxin best detected by intracutaneous injection of rabbits; (4) a lethal toxin, and (5) a gastro-enteric toxin concerned in food poisoning.

METHODS FOR THE IDENTIFICATION OF STREPTOCOCCI

1. Streptococci occur in chains of variable length (Fig. 237); the longest chains are always observed in broth cultures. On solid media the chains are usually short and sometimes in diplococcus formation, requiring differentiation from pneumococci. They are not encapsulated although some types sometimes show capsular-like material when stained by Wright's method.

2. They stain readily and are usually gram-positive. However, gram-negative elements may occur and some saprophytic streptococci are entirely gram-negative in their staining characteristics.

3. On blood agar the colonies are small, grayish, delicately opalescent and usually resemble small droplets of fluid. Surface colonies may be mucoid, smooth, rough or intermediate according to the type. Hemolysis may or may not occur:

    (a) Hemolytic or beta type: zones of complete hemolysis (Fig. 238).

    (b) Nonhemolytic or gamma type: no hemolysis.

    (c) Viridans or alpha type: zones of incomplete hemolysis or greenish methemoglobin around surface colonies.

A streptococcus should not be regarded as nonhemolytic unless it has been subcultured at least twice or grown anaerobically.

4. In broth the growth is never evenly diffuse; turbidity is absent or slight. A finely granular growth occurs with sediment on bottom and sides sometimes difficult to break up.

5. Anaerobic cultures should be made in cases of puerperal sepsis, gangrene of the skin, wound abscesses following operations upon the upper gastro-intestinal tract and appendix, suppurative pneumonitis and empyema for *Str. foetidus*, *Str. putridus*, *Str. anaerobius*, etc. There are many micro-aerophilic types between aerobic and anaerobic types of the genus *Streptococcus*.

6. As a general rule such examinations are sufficient for routine work, the results being reported as "*Streptococcus hemolyticus*" or "*Streptococcus viridans*" as the case may be.

7. If an additional test for hemolysis is required, add 0.5 c.c. of a broth culture

to 0.5 c.c. of a 5% suspension of washed erythrocytes (rabbit preferred), and incubate in a water bath at 37° C. for 2 hours. Examine at intervals for hemolysis.

8. For differentiation from pneumococcus add 0.1 c.c. of clear ox bile to 0.5 c.c. of a pure broth culture and incubate in a water bath at 37° C. for 1 hour, or add 2 drops of a 10% water solution of sodium desoxycholate to 1 c.c. of culture
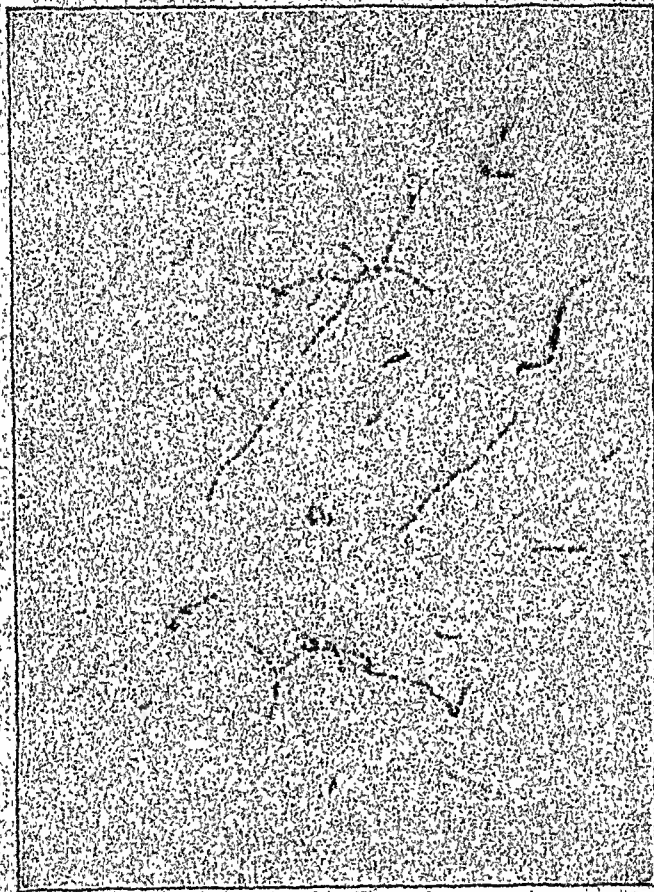
Fig. 237.—Streptococcus Pyogenes (Zinsser)

(Leitson). Streptococci are not soluble while most pneumococci go into solution.

Also inoculate a tube of Hiss' serum water litmus inulin medium and incubate at 37° C. for 24 to 48 hours. Streptococci do not usually change the medium while most pneumococci produce acid.

9. In the older literature hemolytic streptococci were subdivided on the basis of their clinical sources and their fermentation of various carbohydrates into supposed species under the names *Str. pyogenes*, *Str. scarletinae*, *Str. erysipelatis*, *Str. anginosis*, etc. Recent work has shown, however, that these strains are not specific for the disease conditions from which they have been isolated; on the contrary one and the same strain of streptococcus can cause sore throat, scarlet fever, and puerperal fever in different subjects, depending upon individual susceptibility and

the circumstances of infection. It is advisable, therefore, not to perpetuate the errors of the past by reproducing the older classification in these terms. The modern and clinically significant classification is based upon serological grouping and typing.

Grouping of Streptococci by the Precipitin Reaction (Lancefield [1]).—1. Streptococci may be divided in 8 groups, (A, B, C, D, E, F, G and H) by means of the precipitin reaction, using the "C" antigen (carbohydrate) of Lancefield. All of the types in Group A, and an occasional type in the other groups are pathogenic

for man. All of the other groups are either animal pathogens, or are not pathogenic.

2. Add 50 c.c. of N/10 hydrochloric acid to the organisms obtained by centrifuging 50 c.c. of an 18-hour neopeptone broth culture. Boil the suspension for 10 minutes, cool rapidly, and centrifuge. Remove and neutralize the supernatant fluid, centrifuge, and then pour the supernatant fluid into 4 volumes of 95% alcohol. The resulting precipitate is separated by centrifugation, and heated in a boiling water bath until all traces of alcohol disappear. This material is the "C" antigen.

Fig. 238.—Zones of Hemolysis around Colonies of Hemolytic Streptococci

(From Park, Williams and Krumwiede, *Pathogenic Microörganisms*, Lea and Febiger.)

3. Dissolve the antigen in 50 c.c. of saline solution, and then make 3 serial dilutions, decreasing the concentration of the antigen 10 times for each successive dilution. Add 0.2 c.c. of each antigen dilution to successive 0.2 c.c. portions of each unadsorbed group rabbit antiserum. Place in the water bath at 37° C. for 30 minutes, and then in the ice-chest overnight. One or more of the tubes containing the homologous antiserum and the antigen will show a hard white precipitate.

Grouping and Typing of Streptococci by the Micro-Agglutination Technic (Griffith [2]).—1. Streptococci may be divided in 8 groups by this method, the groups being identical with those determined by the precipitin test.

2. Place a loopful of each of the unadsorbed rabbit antisera on the same microslide, each drop being kept separate.

3. Mix a loopful of a thin suspension of an 18-hour culture of the organism with each drop of antiserum, flaming the loop after every operation.

4. Observe under the low power objective. The antiserum of the homologous group will cause the organisms to agglutinate within a few minutes.

5. With few exceptions, only Group A (Lancefield) streptococci will be encountered in the clinical laboratory. Group A consists of 28 or more types, which are designated by arabic numerals (1, 2, 3, etc.).

6. Adsorb each of the 28 rabbit antisera with a heterologous type until it will

[1] *J. Exper. M.*, 1928, 47:91, 469 and 857; ibid., 1933, 57:571.
[2] *J. Hyg.*, 1934, 34:542.

only agglutinate the homologous type. These antisera will then be type specific.

7. Place a loopful of each of the 28 type specific antisera on one or more microslides, keeping each drop numbered and separate. Mix a loopful of the same suspension of organisms which was used for grouping with each of the antisera in turn.

8. Observe under the low power objective. The type specific antiserum of the homologous type will cause the organisms to agglutinate within a few minutes.

Note.—These methods of grouping and typing streptococci relate these organisms to their invasive power, in that the members of Group A are pathogenic for man, and the various types within that group are separate entities as shown by correlation with mouse protection tests and phagocytosis by neutrophil leukocytes.

At present the typing of streptococci has not been developed, however, to the extent where it can be used routinely in the clinical laboratory.

Of the hemolytic streptococci producing disease in human beings, the majority belong to Group A according to the precipitation test, produce a final pH of 4.6 or higher in dextrose broth, fail to ferment sorbitol or to hydrolize sodium hippurate.

Test for Fibrinolysis by Hemolytic Streptococci (Tillet and Garner [3]).—

1. To 0.2 c.c. of oxalated plasma of the patient add 0.8 c.c. of sterile saline solution and 0.5 c.c. of broth culture or sterile filtrate; mix.

2. Add 0.25 c.c. of a 0.25% solution of calcium chloride.

3. Mix and place in a water bath at 37.5° C.

4. The time at which solid coagulation occurs is noted.

5. Continue to observe at intervals and note time at which complete dissolution of the clot (fibrin) occurs. All tests in which the plasma clot is resistant to dissolution after 24 hours' incubation are arbitrarily terminated.

6. The plasma clots of patients recovered from hemolytic streptococcus infections are usually highly resistant to dissolution (fibrinolysis).

Mouse Inoculation Test.—1. Inject a mouse intraperitoneally with 0.1 to 0.5 c.c. of 24-hour broth culture. *Str. pyogenes* and *Str. anginosus* usually produce a fatal septicemia.

2. Inject a mouse intraperitoneally with 1 or 2 c.c. of blood immediately after aspiration from a vein of the patient. Virulent streptococci like *Str. pyogenes*, if present, usually produces a fatal peritonitis and septicemia. Stain smears of heart blood by method of Gram. The presence or absence of capsules aids in differentiation from pneumococci.

METHODS FOR THE IDENTIFICATION OF M. TETRAGENUS

1. This organism is commonly found in the sputum and especially in tuberculosis and other chronic infections.

2. In smears of sputum and secretions it occurs as 4 cocci surrounded by a pseudocapsule (tetrads) while in cultures on ordinary media it usually occurs in pairs and irregular masses with no capsules (Fig. 239).

[3] *J. Exper. M.*, 1933, 58:485.

3. Gram-positive.

4. On agar plates the colonies are circular, white or grayish-white, smooth, glistening and glutinous, often adherent to the medium and difficult to emulsify. On blood agar the colonies are surrounded by a narrow zone of greenish discoloration (alpha type of hemolysis). In broth there is even turbidity with a later thick, glutinous deposit and comparatively clear supernatant medium.
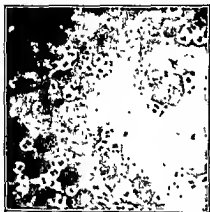
5. These characteristics are usually sufficient for identification. It commonly produces acid in glucose, maltose, lactose and sucrose. No indol; nitrates are reduced.

Fig. 239.—Micrococcus Tetragenus

METHODS FOR THE IDENTIFICATION AND DIFFERENTIATION OF PNEUMOCOCCI

1. If sputum from pneumonia is being examined, smears should be prepared of blood-tinged ("rusty") portions on slides. If cerebrospinal fluid is being examined, centrifuge and prepare smears of the sediment. The pus of empyema or other exudates (nose, ears, etc.) may be prepared in smears.

2. Dry in air; fix by gentle heating and stain by the method of Gram.

3. The pneumococcus is gram-positive and occurs in pairs or, less frequently, in short chains (must be differentiated from streptococcus). The adjacent ends of the cocci are usually rounded while the opposite ends are more pointed or lancet-shaped. A well-marked capsule is present (Fig. 240) which may be retained in cultures on suitable media. Type III pneumococcus may often be identified by very large capsules. Special capsule stains may be employed.

4. With a platinum loop streak out one or two blood agar plates with material to be examined. Incubate 24 to 48 hours. Examine colonies; prepare smears and stain by method of Gram. Or culture by the following method of Avery: Select a tenacious portion of the sputum about the size of a bean, and wash 3

Fig. 240.—Pneumococci with Stained Capsules (Zinsser)

or more times in sterile saline solution. Grind the washed sputum in a sterile mortar with about 1 c.c. of sterile broth to secure a homogeneous suspension. Inoculate a tube of Avery broth with about 0.2 c.c. In the case of spinal, pleural or other fluids inoculate with 1 c.c. Incubate at 37° C. for 5 to 8 hours. If a good growth has

occurred, centrifuge at low speed for 2 minutes to throw down the erythrocytes. Inoculate blood agar plates with the supernatant bacterial suspension and incubate for 24 to 48 hours. With the balance prepare smears and stain by the method of Gram. If pneumococci are present in practically pure culture the suspension may be preserved and used, when occasion arises, for bile solubility, agglutination and precipitation tests.

5. Colonies on blood agar at the end of 48 hours' incubation are usually smooth and circular with the edges sharply raised from the surface of the medium and surrounded with greenish zones of incomplete hemolysis. Prepare smears of colonies and stain by Gram method.

6. Inoculate a tube of glucose hormone broth and a tube of Hiss' serum water litmus inulin medium with pure colonies.

7. Incubate 24 hours. Examine smears stained by Gram method. Pneumococci usually produce acid and coagulation of the inulin medium with a change of color from blue to pink; streptococci do not.

8. If a pure culture is present, conduct a bile solubility test as follows: To about 1 c.c. of the broth culture add 0.2 c.c. of clear sterile ox bile. Prepare a control of 1 c.c. of culture; add 0.2 c.c. of saline solution. Place in water bath or incubator at 37° C. for 2 hours. Note opacity of the tubes. Pneumococci are soluble but streptococci are not. Or add 2 drops of a 10% water solution of sodium desoxycholate to 1 c.c. of culture (Leifson). Pneumococci go into perfect solution in 2 to 5 minutes.

9. Another method for the rapid isolation of pneumococcus from sputum is as follows: (a) Inoculate a mouse intraperitoneally with 1 c.c. of the emulsion of washed sputum prepared as described above. (b) Usually in from 4 to 48 hours, the animal becomes ill and succumbs (the time varies according to numbers and virulence of pneumococci present). (c) When ill kill the mouse or immediately after death, remove the peritoneal exudate aseptically with a capillary pipet. Prepare smears and stain by Gram method. Inoculate glucose hormone broth. Also prepare smears and culture of heart blood. The balance of the peritoneal exudate may be used for agglutination tests as described below for determination of type of pneumococcus.

10. As a general rule such examinations are sufficient for identification of pneumococcus and differentiation from streptococcus.

Serological Types of Pneumococci.—There are three types of pneumococci, designated as Types I, II and III, and a heterogeneous group comprising at least 29 or more types designated as Group IV of which Type VII is the most common. They differ in virulence and immunological reactions. Strains belonging to Group IV are the least virulent and are the ones commonly found in normal throats and are called the "mouth strains." The most virulent are Types II and III; the latter has a tendency to grow in chains and develops a very large capsule and at one time was known as the *Streptococcus mucosus.*

The Neufeld "Quellung" Test for the Rapid Microscopical Differentiation of Pneumococci.—1. Fresh sputum, spinal fluid or pleural fluid should be used,

although if kept on ice, sputum may be typed satisfactorily even when several days old.

2. With a small loop place small flecks of sputum ("rusty" portions preferred) on each of 4 coverglasses. When the sputum is very tenacious, it may be dislodged with another loop or minute sections snipped off with flamed scissors. Only a few
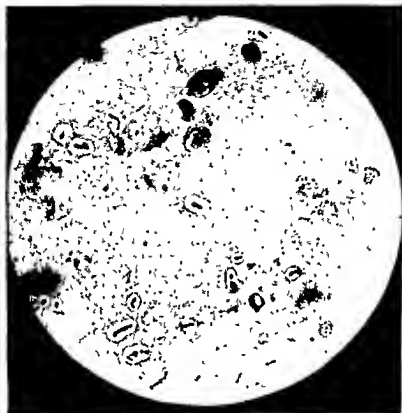
Fig. 241.—The Neufeld "Quellung" Reaction for the Rapid Microscopical Differentiation of Pneumococci (× 1500) Bullowa and Wilcox

(Courtesy of Lederle Co.)

organisms should be used—2 or 3 per field—otherwise a typical reaction may not occur.

3. Add a loopful of Type I, II, III and VII pneumococcus typing sera to each respectively.

4. After mixing, add a loopful of diluted Löffler's methylene blue (1 : 5 dilution in distilled water) to each and mount on hollow-ground glass slides, sealing the edges with vaseline; or the coverglasses may be inverted on ordinary slides and pressed down to make thin layers.

5. Examine under a good microscope with an oil-immersion lens with the light

diaphragm partly closed. An artificial blue light is advantageous. The light should not be too subdued, but of sufficient intensity to prevent shadows and the appearance of artifact halos around the organisms.

6. A positive reaction occurs in a few minutes and is indicated by the appearance of definitely outlined capsules about the pneumococci providing a type is present
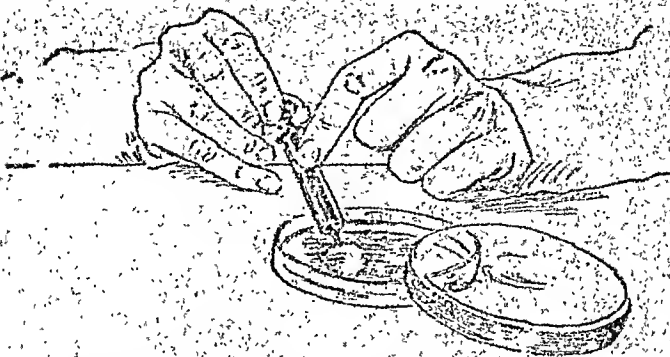
FIG. 242.—WASHING SPUTUM IN SALT SOLUTION
(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

corresponding to the sera employed (Fig. 241). The sharpness of the outline of the capsule is perhaps of more diagnostic value than the degree of swelling. If no reaction occurs immediately, the preparations may be examined again at the end of 10 minutes. If Type III pneumococci are present, the organisms may appear in large masses without definite capsular swelling. In such cases it may be neces-
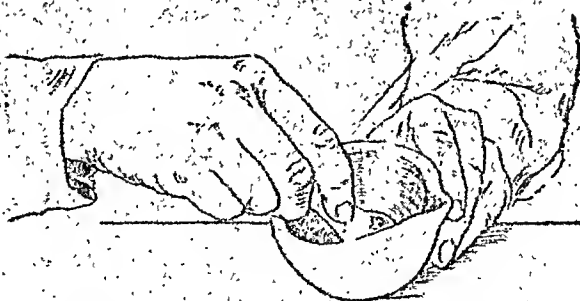
FIG. 243.—EMULSIFYING SPUTUM IN BROTH BEFORE INOCULATION OF MOUSE
(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

sary to dilute the sputum with saline before the tests are conducted. It would appear that Type III serum may sometimes produce a reaction with Type VIII pneumococcus.

Method of Avery, Chickering, Cole and Dochez for Differentiation of Pneumococci.—1. If the sputum is very fluid and has no firm portions, inoculate a mouse directly. Otherwise, take up a firm portion, consisting of not more than

0.5 c.c., in a sterile glass syringe without a needle and wash it three times in st
salt solution. Grind the washed sputum in a sterile mortar, adding from 1.5
2.5 c.c. of broth during the process (Figs. 212 and 213).

2. Inoculate a mouse intraperitoneally with from 0.5 c.c. to 1 c.c. of the dilut
washed sputum (see Fig. 39).

3. Within from four to eight hours, puncture the peritoneum of the mouse w
a sterile needle attached to a syringe and withdraw one or two drops of exuda
Spread this on a slide and stain by Gram's method. If microscopic examinat
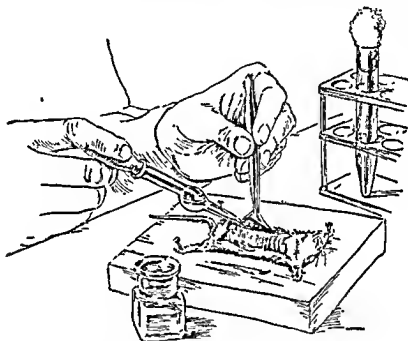
FIG. 214.—COLLECTING PERITONEAL WASHINGS
(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

shows as many gram-positive cocci as may be found in an 18-hour broth cultu
chloroform the mouse and proceed with the test.

4. Since it is desired to recover the organisms in pure culture, carefully obser
sterile precautions throughout the autopsy.

5. When the skin has been laid back, make a short, longitudinal opening in
the abdominal wall (Fig. 214). Take a loopful of the peritoneal exudate a
streak half a blood agar plate. Then enlarge the opening in the abdominal w
and note if the exudate is sticky, suggesting the presence of Type III pneumoco
or *B. mucosus-capsulatus* (Friedländer's bacillus). Using a bulb pipet, wash t
peritoneum thoroughly with from 3 to 4 c.c of salt solution, and put the washin
in a centrifuge tube. Then, with sterile instruments, open the thoracic cavity, an
from the heart's blood, inoculate a tube of pneumococcus broth and streak t

other half of the blood agar plate which has been used for the peritoneal exudate (Fig. 245).

6. Centrifugalize the peritoneal washings at low speed for a few minutes; pour the supernatant suspension of organisms into a second centrifuge tube, and discard the sediment which contains cellular debris from the peritoneum. Centrifugalize the suspension at high speed for from 15 to 20 minutes or until it is perfectly clear. Remove with a pipet the supernatant fluid for a precipitation test (see below) and resuspend the sediment in salt solution for the agglutination test. Perform both
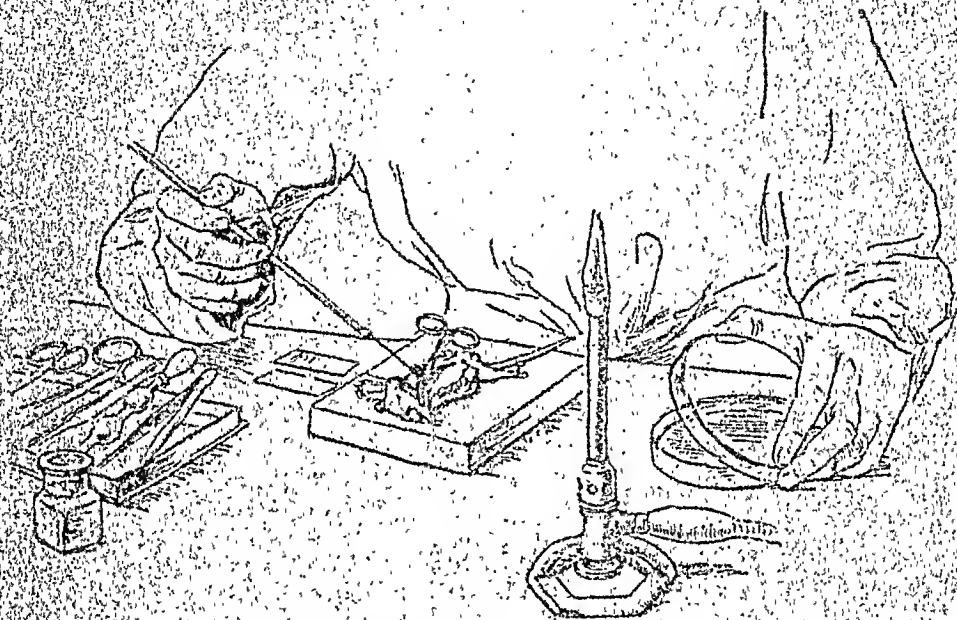
FIG. 245.—HEART BLOOD CULTURE

(From Wadsworth, *Standard Methods*, Waverly Press, Inc., Baltimore.)

tests as a matter of routine, since the former, in some instances, and the latter, in others, has been found to give more prompt and definite results.

7. Use for the routine tests Types I, II, and III antipneumococcus serum, and test, at the same time, the solubility of the organisms in ox bile. Be sure the serum is clear each time before it is used, since the presence of a precipitate would obscure the reaction. If there is a precipitate present, centrifugalize the serum under aseptic precautions. Also be sure that the salt solution used in making the suspensions and serum dilutions is free from sediment.

8. Emulsify the sediment with normal salt solution, making a moderately heavy suspension (should equal at least an 18-hour bouillon culture of pneumococcus).

9. Place 0.5 c.c. of this emulsion into each of 6 small test tubes.

10. Into the first tube add 0.5 c.c. of Type I pneumococcus serum (undiluted); to the second tube add Type II serum (undiluted); to the third tube add Type II serum (diluted 1:20); to the fourth tube add Type III serum (undiluted); to

the fifth tube add a few drops of bile to determine if the emulsion contains chiefly bile-soluble organisms. To the sixth tube add 0.5 c.c. saline (control).

11. Mix well; place in water bath at 37° C. for one hour.

12. Agglutination is indicated by a flocculent appearance in contrast to uniform cloudiness of the control, along with rapid settling of the agglutinated organisms leaving the fluid above clear. The bile tube clears; if not, the suspension is probably not of pneumococci.

13. If agglutination takes place in the tube containing Type I serum and not in any of the other tubes, the strain is Type I; if agglutination occurs in both the tubes containing Type II serum (undiluted and diluted), the strain is a typical Type II; if agglutination occurs only in the tube containing undiluted Type II serum, the strain is an atypical Type II; if agglutination occurs in the tube containing Type III serum, the strain is Type III. If no agglutination takes place in any of the tubes, the strain belongs to Group IV.

Whenever there is an indication of the presence of more than one type of pneumococcus, plate the culture and make an agglutination test with each subculture isolated, in an effort to isolate the types.

Contaminating organisms, especially streptococci, may cause irregular agglutination reactions, but they are not dissolved by the bile. Such cultures should be plated and, if present, obtained in pure culture.

Precipitation Test for Differentiation of Pneumococci.—1. Centrifuge the mouse peritoneal exudate and use the clear supernatant fluid.

2. In a series of 6 small test tubes set up the following:

No. 1: 0.3 c.c. serum Type I (undil.) + 0.3 c.c. supernatant peritoneal washings.

No. 2: 0.3 c.c serum Type I (dil. 1:10) + 0.3 c.c. supernatant peritoneal washings.

No. 3: 0.3 c.c. serum Type II (undil.) + 0.3 c.c. supernatant peritoneal washings.

No. 4: 0.3 c.c. serum Type II (dil. 1:10) + 0.3 c.c. supernatant peritoneal washings.

No. 5: 0.3 c.c. serum Type III (undil.) + 0.3 c.c. supernatant peritoneal washings.

No. 6: 0.3 c.c. serum Type III (dil. 1:15) + 0.3 c.c. supernatant peritoneal washings.

3. Add the supernatant fluid slowly to each tube and look for the formation of a ring of white precipitate at the point of contact. If no immediate reaction occurs, place the tubes in a water bath at 37° C. and observe after 15, 30 and 60 minutes of incubation.

4. After incubation a fibrin web may appear in all the tubes, and care must be taken not to confuse this with a specific reaction.

5. The advantages of this method are: (a) It is not obscured by contaminating organisms; (b) it is highly specific, and (c) it is often applicable when the agglutination test cannot be used.

Sabin Microscopical Method with Peritoneal Exudate of White Mouse.—
1. Three or 4 hours after the injection of a mouse obtain some of the peritoneal exudate by puncture with a capillary pipet.

2. Place 4 small drops on slides.

3. To one add a loopful of saline as a control and to the others a loopful of Type I, II and III immune sera respectively diluted 1:10 with saline solution.

4. Spread the mixtures thinly and allow to dry rapidly; fix with gentle heating.

5. Stain with basic fuchsin (10 c.c. of saturated alcoholic solution plus 90 c.c. of water).

6. Wash with water; dry and examine with oil-immersion lens.

7. A positive reaction is indicated by stained clumps of diplococci. Nonspecific *clumping may be excluded by comparison with the control. As the mouse is not* killed. the test may be repeated if unsatisfactory.

8. This test is also applicable to spinal fluid containing sufficient organisms.

Avery Cultural Precipitation Test for the Differentiation of Pneumococci.
—1. Inoculate 4 c.c. of Avery's medium (meat infusion broth), containing 1% glucose and 5% sterile defibrinated blood, with 0.2 c.c. of diluted, washed sputum or with same amount of fluid sputum. With spinal fluid inoculate with 1 c.c.

2. Mix and incubate for 5 to 7 hours.

3. Make a smear and stain by Gram's method. If a good growth of gram-positive diplococci has occurred, centrifugalize for a few minutes to throw down the blood cells.

4. Transfer 3 c.c. of the supernatant fluid to a sterile centrifuge tube and add 1 c.c. of sterile ox bile.

5. Incubate for 30 minutes to dissolve the pneumococci.

6. Centrifugalize at high speed for 20 minutes.

7. With the clear supernatant bile solution, set up the precipitation test described above.

Methods for the Serological Differentiation of Pneumococci in Blood Cultures.—1. If smears show gram-positive diplococci, presumably the pneumococcus, centrifugalize 10 c.c. at low speed for a few minutes to thrown down the erythrocytes.

2. With the supernatant broth culture of organisms conduct the microscopic agglutination test of Avery, Chickering. Cole and Dochez as described above. Or *it may be used for the Sabin microscopic method as described.*

3. Include the bile solubility test.

Methods for the Serological Identification and Differentiation of Pneumococci in Cerebrospinal Fluid.—1. It is necessary to differentiate between streptococcus and pneumococcus.

2. Make a smear and stain by Gram's method.

3. If a large number of gram-positive diplococci are present, centrifugalize briefly to throw down pus cells and conduct agglutination tests with the supernatant suspension of diplococci.

4. The balance of the fluid may be recentrifugalized at high speed for twenty minutes and the clear supernatant fluid used for a precipitation test.

5. If the diplococci are too few for these direct tests, inoculate a mouse with 0.5 c.c. and conduct tests with peritoneal washing as described above.

6. Or inoculate 4 c.c. of Avery's medium with 1 c.c. of fluid and conduct the test as described above.

7. Include a test for bile solubility.

METHODS FOR THE IDENTIFICATION OF THE GONOCOCCUS

1. Materials submitted for examination for the gonococcus (*Neisseria gonorrhoeae*) are usually purulent secretions from the genital tract of both sexes, the vagina of children and the conjunctivae (especially of infants).

2. Well-prepared smears stained by the method of Gram and carefully examined are still mainly relied upon for bacteriological diagnosis. There is no specific differential stain for the gonococcus.

3. Typical gonococci are gram-negative. Duplicate smears stained with Löffler's methylene blue are advisable for morphology but should not be relied upon alone for identification.

4. They are usually arranged in pairs, with adjacent sides flattened or slightly concave, resembling a pair of kidney beans. They are not encapsulated.

5. In the pre-acute stage before the exudate becomes profuse, the organisms may be extracellular but become intracellular (Fig. 216) during the acute stage when the exudate is at its height. At this stage it is common to find many organisms gathered within one leukocyte while other cells in the immediate neighborhood have none. Later, when the infection becomes more chronic, the organisms become less numerous and are extracellular. In gonococcal conjunctivitis the organisms may occur in or upon epithelial cells.

Fig. 216.—Gonococci within Pus Cells (Zinsser)

6. A *positive* report may be rendered when smears show large numbers of pus cells with gram-negative intracellular diplococci or typical cultures or both. Smears of urethral discharge from early cases and likewise from chronic cases (gleet) showing pus cells, many extracellular gram-negative diplococci and at least a few typical intracellular organisms, may be also reported as positive. Special care is required in reporting on vaginal smears because other diplococci resembling gonococci are encountered more frequently than in the urethra; here cultures should be made, especially in medicolegal cases.

A positive report may also be rendered in smears showing 50% or more polymorphonuclear pus cells with many extracellular and occasional intracellular gram-negative diplococci of typical morphology.

7. A *suspicious* report may be rendered in cases with discharge when smears show many polymorphonuclear pus cells but no intracellular diplococci.

8. A *negative* report may be rendered when smears show only a few polymorphonuclear pus cells with no suspicious intracellular diplococci and no clinical evidences of disease. In any positive, doubtful, or suspicious case, smears should be made once a week until a negative is obtained to be followed by 3 more well-made smears at intervals of about 3 days before one is prepared to render final report of negative.

9. The gonococcus is ordinarily difficult to isolate and cultivate and especially in chronic infections. The material may be collected on a sterile swab and the latter mixed well in 1 or 2 c.c. of sterile ascites fluid or broth in a test tube. With this suspension prepare surface streakplates. Many different media have been proposed. A satisfactory medium is prepared by heating 1.5 or 2% meat infusion agar carrying 0.5% glucose with a final pH of 7.3 to 7.6 until melted: cool to 70° C. and add sterile defibrinated blood of man, rabbit or horse (preferred) to give about 5%. Glucose agar 4 parts with 1 part of sterile ascites fluid in plates is also recommended.

10. After inoculation the plates should be incubated at 34 to 35° C. and preferably in a sealed jar carrying 10% carbon dioxide.

11. At the end of 48 to 72 hours examine. Colonies of gonococci are usually small, delicate, grayish, opalescent, slightly convex and slightly elevated with scalloped edges, sticky and mucoid. Gently flood the surface of the plate with 1 c.c. of a *freshly* prepared 1% aqueous solution of dimethyl-para-phenylene-hydrochloride and pour off in 3 to 5 minutes. Colonies which turn pink in this time so that they can be seen with the naked eye are then picked off for staining (Gram) and sugar fermentation tests. This oxidase reaction is not specific for the gonococcus, as, among others, *B. anthracis*, *B. pyocyaneus* and *B. subtilis* give the reaction, but it is very helpful.

12. Prepare subcultures of the pink colonies on rubber-stoppered slants of 1% glucose, lactose, sucrose, and maltose ascitic agar to which Andrade's indicator is added. Incubate at 35° C. for 48 to 72 hours. The gonococcus produces acid (no gas) only with glucose. Or prepare subculture on the sugar agars without the indicator and after 2 or 3 days' incubation, allow a drop of 0.02% aqueous solution of phenol red to flow over the surface (Bayne-Jones). A positive reaction is indicated by a change of color to yellow confined to the surface or to a thin superficial layer.

METHODS FOR THE IDENTIFICATION OF THE MENINGOCOCCUS

1. Materials submitted for examination for the meningococcus (*Neisseria intracellularis*) are usually spinal fluids, blood cultures and cultures of the nasopharynx.

2. In smears of spinal fluid, the meningococcus is usually arranged in pairs bearing a close resemblance to the gonococcus in morphology. When thoroughly decolorized they are gram-negative organisms and unless well decolorized may

appear gram-positive with the possibility of being mistaken for the pneumococcus. They are not encapsulated.

3. Early in meningococcus meningitis the number of organisms present in smears may be very few and largely extracellular, requiring careful examination. Later they become more numerous and largely intracellular (Fig. 217). As a general rule the finding of intracellular and extracellular gram-negative, coffee-bean shaped diplococci in spinal fluid justifies the immediate presumptive diagnosis of

FIG. 217.—MENINGOCOCCUS IN SPINAL FLUID (Zinsser)

meningococcus meningitis, with the prompt administration of antimeningococcus serum.

4. On blood agar plates incubated at 37° C. for 48 hours, the colonies are small, moist, convex, elevated, colorless, transparent, circular and nonhemolytic. On serum or ascites agar plates they are round, convex, bluish-gray, smooth and glistening with entire edges. Later they increase in size, become more yellow and opaque and may show a granular center.

5. Prepare smears and stain by the Gram method. Gram-negative diplococci of varying size and shape are usually meningococci. Transplant to slants of blood ascites agar or sheep serum dextrose agar. Incubate 24 hours and examine.

6. Test for fermentation by inoculating tubes of 1% dextrose, maltose, levulose and sucrose agar to which Andrade's indicator has been added. Incubate for 48 to 72 hours. The meningococcus produces acid (no gas) with dextrose and maltose.

7. The following *presumptive slide agglutination test* may also be conducted for

confirmatory purposes: (a) On a clean glass slide place 1 drop of polyvalent antimeningococcus serum diluted 1:10; on a second, 1 drop of normal horse serum 1:10 and on a third, 1 drop of normal saline solution. (b) With a small platinum loop mix a portion of suspected colony in each of the three drops. (c) Allow to stand at room temperature for 5 to 15 minutes and examine. (d) Agglutination on the first slide is sufficient for a presumptive diagnosis of "meningococcus, type undetermined." (e) Allow the films to dry and stain by Gram method to verify morphology, staining characteristics and agglutination.

Rapid Method for the Detection of Meningococcus Carriers (Olitsky).—
1. Material from the *nasopharynx* should be collected on a sterile cotton swab on a heavy wire, the last 2 cm. of which is bent at an angle of about 40 degrees, being careful to avoid possible contamination due to contact with secretions from the mouth or throat.

2. Inoculate warm plates of blood agar or serum agar and incubate at 37° C. for 24 to 48 hours.

3. Examine for colonies of meningococci. Prepare smears and stain by Gram method.

4. Transfer characteristic colonies of gram-negative diplococci to the following medium: add 1 c.c. of unheated, sterile, clear normal horse serum to 9.5 c.c. of 1 per cent glucose broth and distribute 1 c.c. amounts in small sterile tubes.

5. Incubate 12 to 24 hours and examine. The meningococcus produces a faintly turbid growth with slight sediment which emulsifies readily when shaken. Prepare smears and stain by Gram method.

M. flavus, M. perflavus, M. sub-flavus, M. flavescens and *M. pharyngis siccus* show gram-negative diplococci with firm agglutination by the normal horse serum with a clear supernatant fluid.

M. catarrhalis shows gram-negative diplococci with a dense turbidity often with a pellicle on the surface.

Staphylococci are gram-positive and produce a dense turbidity, an agglutinated sediment and often a pellicle.

Streptococci are gram-positive and produce a granular sediment with a clear or slightly turbid supernatant fluid.

B. influenzae fails to grow.

6. To such tubes as are suspicious of meningococci add 0.1 c.c. of a 1:10 dilution of high-titer polyvalent antimeningococcus serum.

7. Incubate in a water bath (not an incubator) at 37° to 38° C. for 2 hours.

8. Prepare smears of tubes showing agglutination and stain by Gram method. Agglutinated masses of gram-negative diplococci are presumptive evidence of meningococcus.

9. Transplants may be made to slants of blood agar. Incubate 24 hours. Prepare smears and stain by Gram. Transplant cultures showing gram-negative diplococci to slants of dextrose, maltose, levulose and sucrose agar to which *Andrade's* indicator has been added. Incubate 24 to 48 hours and examine for production of acid (gas is not produced).

TABLE III

Production of Acid	Dextrose	Maltose	Levulose	Sucrose
Meningococcus	+	+	—	—
M. catarrhalis	—	—	—	—
M. pharyngis siccus	+	+	+	+
M. perflavus	+	+	+	+
M. flavus	+	+	+	—
M. sub-flavus	+	+	+	—
M. flavescens	—	—	—	—

Serological Types of Meningococci.—By agglutination and agglutinin absorption tests meningococci have been divided into Types I, II, III and IV. Types II and IV are closely related and often grouped together. In the United States the typing of meningococci is not usually requested since polyvalent sera are employed in treatment.

Microscopical Agglutination Test for the Differentiation of Meningococci.—I. Arrange 6 small test tubes.

2. Into Nos. 1, 2, 3 and 4 place 0.5 c.c. of specific agglutinating sera for Types I, II, III and IV respectively; the dilutions to employ will depend upon the respective titers furnished with the sera.

3. Into No. 5 place 0.5 c.c. of a 1:25 dilution of normal horse serum (control).

4. Into No. 6 place 0.5 c.c. of saline solution (control). .

5. Prepare a heavy uniform suspension of the meningococcus cultivated on blood or serum dextrose agar and place 0.5 c.c. in each tube.

6. Mix well and place in a water bath at 55° C. for 3 hours. If desirable, they may be then placed in a refrigerator overnight before the readings are made.

7. If the organism is a meningococcus, agglutination should occur in the tube carrying the homologous serum. Inagglutinable strains are sometimes encountered.

8. If the organism is from the nasopharynx and agglutination occurs in all of the first 5 tubes (No. 6 is a control) the test is unsatisfactory as nonpathogenic diplococci found in this region are frequently strongly agglutinated by normal and immune horse sera in final dilutions of 1:50.

METHODS FOR THE IDENTIFICATION OF M. CATARRHALIS

1. M. catarrhalis (Neisseria catarrhalis) occurs principally in the respiratory tract and may be encountered in cultures of the nose, throat and sputum. It is commonly regarded as but feebly pathogenic.

2. In smears of fresh material it occurs as a gram-negative diplococcus larger than the meningococcus and at times arranged in tetrads or small groups. In sputum the diplococci are shaped like coffee-beans, and may be both intra- and extracellular. They are not encapsulated.

3. The organism grows much more luxuriantly than the gonococcus and more freely than the meningococcus. On plain or blood agar the colonies at the end of 24 hours' incubation are convex, whitish-gray and glistening; after longer incuba-

tion they become more elevated, opaque, slightly brownish in the center with wave-like periphery, coherent, tenacious, difficult to emulsify and auto-agglutinable in saline solution. In broth there is usually no turbidity with a coarsely granular sediment difficult to break up by shaking; a pellicle may form. The organism is aerobic and will grow at 22° C. whereas the meningococcus and gonococcus require a higher temperature.

4. Sugar fermentation tests are usually required for final identification and differentiation from the meningococcus, *M. flavus*, *M. pharyngis siccus* and other gram negative diplococci occurring in the nasopharynx and sputum. For this purpose inoculate rubber stoppered tubes of dextrose, maltose, levulose and sucrose agar, to which the Andrade indicator has been added. Incubate 24 to 48 hours. *M. catarrhalis* does not produce acid or gas with any of these sugars.

METHODS FOR THE IDENTIFICATION OF B. ANTHRACIS

1. The "malignant pustule" or anthrax of the skin produced by *B. anthracis* may be mistaken clinically for a simple furuncle or carbuncle.

2. It is usually encountered among workers in hides, hair and wools but may be derived from shaving brushes or other articles.
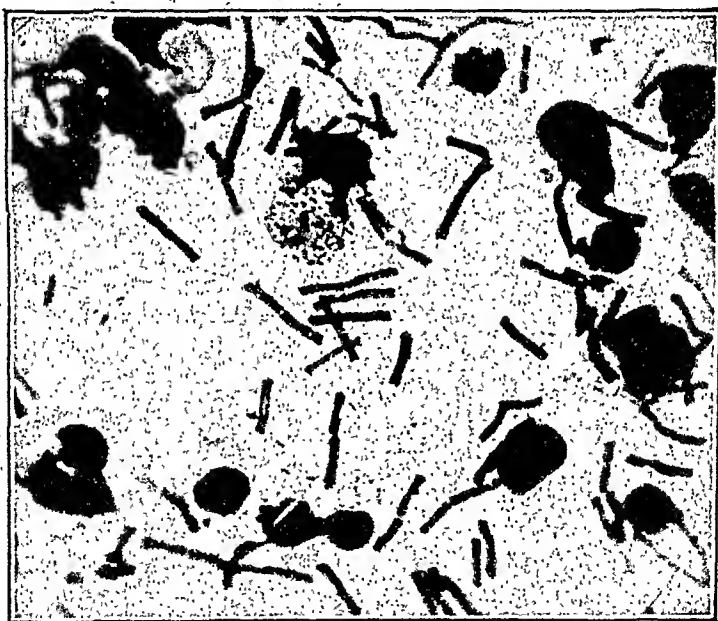
Fig. 248.—ANTHRAX BACILLI
Smear of spleen of animal dead of anthrax (Zinsser)

3. Great care must be exercised in handling material, cultures, etc. Be sure to thoroughly disinfect pus, glassware, etc., before discarding.

4. Septicemia may occur and blood cultures should be included routinely.

5. Anthrax of the bronchi may occur ("wool sorter's disease") in which case the bacillus occurs in the sputum.

6. Anthrax of the intestinal mucosa may also occur with the bacillus in the feces.

7. Prepare smears and cultures of the lesion and particularly of any blister fluid that may be obtained. Blood or plain glucose agar slants or plates may be employed. Avoid rough manipulation of the lesion which might spread the infection.

8. Stain smears by Gram method. The presence of scattered large bacilli with square ends is suspicious. Occasionally short chains are seen (Fig. 218). The bacilli are gram positive if not decolorized too long. They may be encapsulated.

9. Incubate the cultures for 24 hours and examine. Growth is rapid. The colonies are dull, grayish-white, flat and spreading with irregular borders which when viewed under the lower power of the microscope have a Medusa-head appearance due to filamentous interlacing chains of bacilli. Examine smears stained by Gram method; spores are produced.

10. Examine for motility; *B. anthracis* is not motile.

11. Inoculate a Löffler blood serum slant and incubate 24 to 48 hours. *B. anthracis* produces no liquefaction or but very slight.

12. If in doubt, inoculate a young guinea-pig subcutaneously or intraperitoneally with 1 c.c. of a 24-hour broth culture or with a suspension in saline solution. *B. anthracis* usually produces a fatal septicemia in 12 hours to 2 or 3 days with the presence of organisms in the heart blood, spleen, liver and other organs. Prepare smears of blood and spleen; stain by Gram. Also prepare cultures on plain or blood agar. If an early diagnosis is desired, the suspected material may be inoculated subcutaneously into guinea-pigs, mice or rabbits without waiting for the isolation of pure culture.

13. *B. anthracis* must be particularly differentiated from *B. subtilis;* also from *B. mycoides* and *B. mesentericus:*

TABLE IV

	Motility	Blood Serum	Milk	Virulence for Guinea-pigs
B. anthracis	Absent	No or slight digestion	Acid and coagulation	Virulent
B. subtilis	Present	Digestion	Alkaline	Nonvirulent
B. mycoides	Present	No digestion	Acid; no coagulation	Nonvirulent
B. mesentericus	Present	Slight digestion	Acid; no coagulation	Nonvirulent

14. If present in mixed culture, isolation is facilitated by heating a broth culture at 60° C. for 20 minutes and then plating.

15. A positive diagnosis of anthrax may be reported if the specimen contains a gram-positive, square-ended, chain-producing, spore-forming, nonmotile bacillus producing characteristic Medusa-head colonies on agar. Confirmatory tests are positive precipitation and virulence for young guinea-pigs, rabbits or mice.

Precipitin Test.—1. Prepare a suspension of a 24-hour agar slant culture in 5 c.c. of saline solution.

2. Keep for 2 hours at room temperature and filter.

3. In a series of small test tubes place 1 c.c. of undiluted. 1:10 and 1:20 dilutions of antianthrax serum.

4. Carefully overlay with 1 c.c. of filtrate.

5. A positive reaction is indicated by a ring of precipitation within 15 minutes. Other aerobic, spore-bearing bacilli may give a reaction with undiluted serum.

Ascoli Test.—The Ascoli test for the detection of anthrax bacilli in meat may be conducted as follows:

1. Macerate the tissue with 5 to 10 parts of saline solution or 1:1000 acetic acid and boil for 15 minutes.

2. Centrifuge and filter through paper.

3. Place 0.5 c.c. of antianthrax serum of good precipitating titer in a test tube and overlay with 0.5 c.c. of clear filtrate.

4. Stand at room temperature for 15 minutes. A positive reaction is indicated by a white ring of precipitation.

Blood Cultures.—It is always advisable to culture 10 to 20 c.c. of blood in 150 c.c. of plain broth. The bacilli may be present and especially in some cases just before death. When positive they are usually present after an incubation of the culture for 24 to 48 hours.

Isolation from Hair or Bristles.—1. Rub up the suspected material in saline solution.

2. Heat one half at 80° C. for 30 minutes to kill nonspore-forming contaminants.

3. Centrifuge both portions.

4. Prepare four agar plates of both sediments.

5. Inoculate mice and guinea-pigs with both.

6. Study colonies (special attention to deep ones).

METHODS FOR THE IDENTIFICATION OF B. SUBTILIS

1. This organism, commonly known as the "hay bacillus." is nonpathogenic but is occasionally found as a saprophyte in old sinuses and infected wounds. It is also a common contaminant of culture media and may be mistaken for *B. anthracis.*

2. It grows readily and luxuriantly on all ordinary culture media. The colonies are large and spreading with irregular margins due to interlacing chains of bacilli. It is aerobic and in broth grows largely on the surface with a pellicle which later may drop to the bottom of the tube. It rapidly liquefies both gelatin and Löffler's blood serum medium.

3. The organism is gram-positive and occurs as straight rods (Fig. 249) in chains. Spores are found usually slightly nearer one pole than the other but only in the chains.

4. It is actively motile whereas *B. anthracis* is nonmotile. For further differentiation see above under Methods for the Identification of *B. anthracis.*

METHODS FOR THE IDENTIFICATION OF B. DIPHTHERIAE

1. Diphtheria is usually an infection of the upper respiratory tract (nose, fauces, or larynx) with *B. diphtheriae* (*Corynebacterium diphtheriae*). Laryngeal diphtheria is also known as "membranous croup." It is important to make smears and cultures (preferred) with much care as otherwise they may prove negative for the bacilli and misleading.

2. If smears of the exudate have been made, stain with Löffler's methylene blue.

Fig. 249.—Bacillus Subtilis
(Hay bacillus)

Cultures are much more reliable. In direct smears the bacilli are larger and atypical. Smears may be negative in cases giving positive cultures and should not be relied upon alone for bacteriological diagnosis.

3. The bacilli are gram positive. Löffler's methylene blue is the best routine stain. There is no specific stain for differentiation from *B. hoffmanii* and diphtheroid bacilli. Neisser's stain may be used for bringing out more sharply the metachromatic granules.

4. Cultures should be made on slants or plates of Löffler's blood serum medium. Blood agar is next best. Cultures should be incubated at 35° to 37° C. for 18 to 24 hours. At higher temperatures the bacilli become smaller and atypical. Cultures

may be examined as early as 8 to 12 hours but the bacilli are larger, more solid and more difficult to recognize.

5. On Löffler's medium the colonies are small, circular, grayish or creamy white, convex and smooth. On blood agar their appearance is the same; some strains show narrow zones of hemolysis. On potassium tellurite medium containing blood
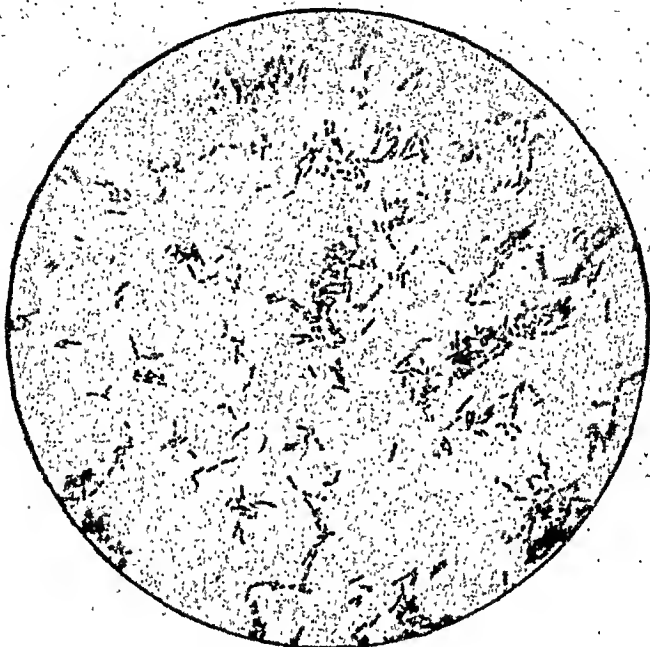
FIG. 250.—DIPHTHERIA BACILLI (Wood)

and tryptic digest, the colonies are black because the bacillus reduces the salt. This medium is recommended as an aid in identification.

6. The organism is aerobic. In broth a fine pellicle may develop on the surface with moderate turbidity and collection of fine granules on the sides of the tube, with a powdery deposit.

7. Prepare smears and stain with methylene blue. Be sure to pass the loop lightly over the entire surface of the slant in order not to miss isolated colonies. The smear will often show a pure or almost pure culture, owing to the fact that in the first 18 to 24 hours the bacilli tend to outgrow other organisms.

8. Diphtheria bacilli are slender, straight or slightly curved rods (Fig. 250) occurring in three principal morphological types, which Wesbrook and others have subdivided into many subvarieties:

(a) *Granular* or *beaded* types, which are most typical and likely to be most virulent

(b) *Segmented* or barred types, encountered less frequently but likewise likely to be virulent

(c) *Solid* types, which may be long or short and likely less virulent. These bacilli are readily mistaken for *B. hoffmanii* and diphtheroid bacilli.

9. Any of these, and especially the granular and segmented types, may have swollen ends producing the typical club-shaped bacilli. They are not encapsulated, do not form spores and are nonmotile.

10. It is usual and characteristic of diphtheria bacilli to lie at various angles to one another forming V or Y shapes, which when clumped give the appearance of Chinese letters. They do not occur in palisade formation or in chains.

11. With experience *B. diphtheriae* may be readily identified by these means. But it is not always possible to safely differentiate the solid types from *B. hoffmanii* and diphtheroid bacilli. Sugar fermentation and guinea-pig virulence tests are sometimes required.

12. The true diphtheria bacillus produces acid but no gas in dextrose, maltose, and dextrin. Hiss serum water litmus medium with 1% of these sugars may be employed for the tests.

Subcutaneous Test for Virulence.—1. If the culture on a slant of Löffler's medium appears to be pure, wash it off with 10 c.c. of sterile saline solution, emulsify and inject a 250 to 300 gram guinea-pig (not heavier) subcutaneously with 4 c.c. in the median abdominal line. Inject a second pig with the same amount plus 1 c.c. of antitoxin (100 to 500 units).

2. If the culture is not pure, first isolate the diphtheria bacilli by the "streak" method on plates of Löffler's blood serum, ascitic or blood agar.

3. Inoculate a tube of glucose broth with several different colonies. If one colony is used, there is danger of picking up a nonvirulent organism and securing a negative result even though virulent bacilli are actually present in the original culture on the slant.

4. Incubate at 37° C. for 48 hours, keeping the tube in a slanted position to give the culture as much oxygen as possible.

5. Examine for purity. Inject a 250 to 300 gram pig subcutaneously in the median abdominal line with 2 c.c. of culture. Inject a second pig with the same amount plus 1 c.c. of antitoxin (100 to 500 units).

6. Observe for at least 4 days. The control animal by either method should survive and show no edema at the site of inoculation.

7. Virulent bacilli by either method will kill the pigs within four days with marked hyperemia of the suprarenal glands. Or the pigs will be sick and show marked edema at the site of inoculation; this is also a positive result even though the pigs may not succumb in four days (they are likely to develop paralysis of the hind legs later on).

Intracutaneous Tests for Virulence.—1. The advantage of this method is that two or three cultures can be tested with one guinea-pig or rabbit. It is, however, less reliable than the subcutaneous tests.

2. Denude an area of abdominal skin by plucking out the hair for injection of each culture.

3. Emulsify a 24-hour growth of a Löffler slant in 10 c.c. of sterile saline solution.

4. Inject 0.15 c.c. intracutaneously.

5. Virulent bacilli produce a positive reaction of definite local inflammation in 24 hours which goes on to superficial necrosis in 48 to 72 hours.

6. A control animal may be inoculated in the same manner, but receiving an intraperitoneal injection of 100 to 500 units of antitoxin. It should show no lesions.

METHODS FOR THE IDENTIFICATION OF B. XEROSIS

1. This is the special name given to the diphtheroid bacillus of the eye.

2. The bacillus may be found on the normal conjunctivae; it sometimes produces a low-grade chronic conjunctivitis.

3. Smears of conjunctival secretion stained by Löffler's methylene blue or Gram's method show solid bacilli of typical grouping and morphology.

4. Cultures on Löffler's blood serum or blood agar show luxuriant growths of large. solid, gram-positive bacilli of typical grouping.

5. Cultures are nonvirulent for guinea-pigs.

6. Cultures usually ferment some sugars in Hiss's serum water medium, especially saccharose and dextrose but not dextrin.

METHODS FOR THE IDENTIFICATION OF B. HOFFMANII

1. *B. hoffmanii* is commonly found in the normal nose. It is now regarded as a separate species rather than a nonvirulent diphtheria bacillus. It is nonpathogenic.

2. The bacillus is shorter and thicker than *B. diphtheriae;* usually straight and slightly clubbed at one end (Fig. 251).

3. Stained with Löffler's methylene blue, the bacilli are solid but occasionally show an unstained transverse band through the middle which may give them a diplococcoid appearance. No polar bodies; no spores; not encapsulated; nonmotile; gram-positive.

4. The organism grows more luxuriantly than *B. diphtheriae.* The colonies are larger, less transparent and whiter. In broth there is less turbidity and less tendency to form a pellicle.

5. Sugar fermentation tests are usually required for final identification. Inoculate a pure culture in Hiss serum water litmus medium carrying 1% of dextrose, saccharose and dextrin. *B. hoffmanii* produces no acid and no gas with these or other sugars.

6. The bacillus is nonvirulent for guinea-pigs.

Fig. 251.—BACILLUS HOFFMANNI (Wood)

METHODS FOR THE IDENTIFICATION OF DIPHTHEROID BACILLI

1. Diphtheroid-bacilli are widely distributed, commonly occurring in the normal nose and throat as well as on the skin, in lymphatic glands, ascitic fluid, etc.

2. They are commonly found in the pus of chronic infections and especially those involving bone (otitis media, wounds, fistulae, etc.) as secondary invaders.

3. They are nonvirulent, occurring in cultures of the nose and throat. They are readily mistaken for *B. diphtheriae* with unnecessary prolongation of quarantine following recovery from diphtheria.

4. The bacilli are usually long or short solid types but often showing metachromatic granules.

5. These organisms grow on plain agar and much more luxuriantly than *B. diphtheriae*. The colonies are large, white and glistening.

6. The guinea-pig virulence test is the only reliable means for differentiation from *B. diphtheriae*.

7. They may produce acid with dextrose and dextrin in Hiss serum water litmus medium.

METHODS FOR THE IDENTIFICATION OF B. INFLUENZAE

1. *B. influenzae* (*Hemophilus influenzae*) is not now usually regarded as the cause of influenza since it appears that a filtrable virus is the primary agent, but it is at least an important secondary organism and at times the primary cause of meningitis, pneumonia and arthritis.

2. It may be found in the nasopharynx of healthy persons.

3. The organism occurs as a very small, short, moderately thick rod, sometimes almost coccal (Fig. 252) but is highly pleomorphic and may occur in long threads or filamentous forms, especially in spinal fluid.

Fig. 252.—Bacillus Influenzae (Zinsser)

Bipolar granules are frequently seen.

4. *B. influenzae* is gram-negative; not encapsulated; does not form spores; is nonmotile.

5. It is best stained with dilute carbolfuchsin or Giemsa's stain.

6. Smears of spinal fluid (usually prepared of sediment after centrifuging) are of great value in diagnosis but direct smears of the nasopharynx are not worth while.

7. Being a strict parasite it requires for cultivation the presence of accessory substances or "growth factors," of which two, X and V, have been distinguished. The X factor, which is thermostable, is associated with hematin and with less well-defined substances present in fruits, potato and other vegetables containing iron. The V factor, which is thermolabile, is found in blood, potato and yeast and resembles vitamin C.

8. Satisfactory media are "chocolate agar," fresh blood agar (human or rabbit blood preferred) and Avery's sodium oleate agar (pH 7.2).

9. Sputum should be obtained from the lower passages and washed in sterile water. Select one or more solid particles and streak on plates. In the case of spinal fluid culture at least 1 c.c. or 0.5 c.c. of sediment. Swabs of the nasopharynx may be streaked on plates.

10. Cultivate 24 hours at 37° C. The colonies are pinpoint in size, smooth, circular, transparent and homogeneous with edges entire and emulsifying readily.

11. Prepare smears and stain by Gram method. Transplant colonies to slants of plain and "chocolate agar." *B. influenzae* will not grow on the former.

12. It is a difficult organism to identify until considerable experience has been gained. In spinal fluid, however, it is readily identified by these characteristics. Most strains produce acid (no gas) with levulose, galatose, dextrose but not with lactose or mannitol. All strains are negative on mannitol and lactose.

METHODS FOR THE IDENTIFICATION OF B. PERTUSSIS

1. It is difficult to isolate *B. pertussis* (*Hemophilus pertussis*) of Bordet-Gengou, even under the most favorable circumstances, because it grows slowly.

2. Good results are secured by culturing the thick viscid pellets of sputum obtained during the early or catarrhal stage as the secretions of the nose usually give negative results. The "cough plate" method devised by Chievitz and Meyer is preferred.

3. The examination of stained smears of sputum is hardly worth while as the bacillus cannot be differentiated from *B. influenza* by morphology alone.

4. *B. pertussis* requires neither the X nor V factors for its growth and the best medium is the glycerin-potato-blood agar of Bordet-Gengou or modifications of it with a pH of about 5.0 as it inhibits the growth of *B. influenza*. Mueller believes that better results are obtained by leaving the medium unadjusted which gives a pH of 6.1 to 5.8.

FIG. 253.—BACILLUS PERTUSSIS

Organisms from forty-eight-hour culture on Bordet-Gengou medium.

5. Pellets of thick tenacious mucus should be taken up on a platinum loop, washed two or three times in sterile saline and streaked on plates.

6. With infants and young children from whom sputum may not be obtainable, open plates of medium may be held 5 or 6 inches in front of the mouth during a paroxysm of coughing or forced expiration ("cough plates").

7. Incubate 48 to 72 hours. The colonies are characteristic being small, dome-shaped, moist and pearly-white surrounded by a narrow darkened zone of hemolysis.

8. Prepare smears and stain by the method of Gram. Best results are obtained with phenol-toluidine blue (Bordet-Gengou) or phenol-methylene blue. It is gram-negative and does not form spores.

9. The bacillus occurs as small ovoid rods (Fig. 253) sometimes bipolar and resembling *B. influenzae*. The forms seen in cultures are often larger than those seen in smears of mucus and filaments are sometimes produced.

10. *B. pertussis* does not ferment any carbohydrate, does not reduce nitrates and forms no indole.

METHODS FOR THE IDENTIFICATION OF THE KOCH-WEEKS BACILLUS

1. The Koch-Weeks bacillus (*Hemophilus conjunctivitidis*) causes acute contagious conjunctivitis commonly known as "pink eye."

2. Diagnosis is best made by the examination of smears of conjunctival secretions stained by the method of Gram.

FIG. 254.—CONJUNCTIVAL SMEAR SHOWING KOCH-WEEKS BACILLI

P, intracellular bacilli

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co.)

3. The organism occurs as a small gram-negative bacillus slightly larger than *B. influenzae*. It is usually intracellular but also extracellular (Fig. 254). In acute cases the bacilli are present in large numbers but are so small that they may be overlooked. They tend to occur in shoals near pus cells. Smears stained with dilute carbolfuchsin or phenol toluidine blue are sometimes to be preferred.

4. As a general rule such findings are sufficient for diagnosis.

5. If cultures are desired, inoculate plates of fresh blood agar as both growth factors, X and V, are required. Ascitic-glycerin agar is also suitable. Incubate for 48 hours. Examine for small, dewdrop colonies of gram-negative bacilli. The bacillus does not ferment the sugars.

METHODS FOR THE IDENTIFICATION OF THE MORAX-AXENFELD BACILLUS

1. The Morax-Axenfeld bacillus (*Hemophilus lacunatus*) produces a type of subacute or chronic angular conjunctivitis.

2. Prepare smears of the conjunctival secretions and stain by the method of Gram.

3. The organism appears as short, thick bacilli, usually in the form of two bacilli placed end to end, but infrequently singly or in short chains. Their ends are distinctly rounded, their centers slightly bulging, giving the bacillus an ovoid form (Fig. 255). They are gram-negative; are not encapsulated, and they do not form spores.

4. Such findings are usually sufficient for diagnosis.

5. If cultures are made, use alkaline agar enriched with serum, blood or ascitic fluid. The organism does not require the X or V factors. Incubate at 37° C. for 48 hours. The colonies are gray and at first almost invisible. On Löffler's blood

serum colonies appear as small indentations or erosions due to liquefaction of the medium from which the name "laennatus" is derived.

6. The bacillus produces acid but no gas with dextrose and mannitol but not with maltose or lactose.

METHODS FOR THE IDENTIFICA-
TION OF THE ZUR NEDDEN
BACILLUS

1. This bacillus is believed to some-times produce corneal ulcers.

2. It is very small, often slightly curved and generally occurs singly as it does not form chains.

3. Prepare smears and stain by method of Gram. It is gram-negative, often staining poorly at the ends. It is not encapsulated.

4. It grows readily on ordinary culture media. Upon plain or blood agar

FIG. 255.—MORAX-AXENFELD DIPLOBACILLUS (Zinsser)

the colonies are transparent, rounded, raised, granular and slightly fluorescent with a tendency to confluence. Gelatin is not liquefied; milk is coagulated; indole is not produced. On dextrose culture media it will produce acid but will not produce gas.

METHODS FOR THE IDENTIFICATION OF THE BACILLUS OF DUCREY

1. The bacillus of Ducrey (*Hemophilus ducreyii*) is regarded as the cause of chancroidal infection ("soft chancre").

2. The best method of examination consists in aspirating an unopened bubo and preparing smears and cultures of the pus. When buboes are not present, material may be scraped from the base of the ulcer or from beneath its overhanging edge with a stiff wire.

3. Stain the smears by the method of Gram.

4. The bacillus is difficult to cultivate; the method of Teague and Deibert is recommended: (1) Bleed a rabbit aseptically from the heart and distribute 1 c.c. amounts in small sterile test tubes. Allow to clot, heat for 5 minutes at 55° C. and keep in refrigerator. (2) With sterile stiff iron wire bent at one end, secure pus by gently rubbing over the base of the ulcer or under its undermined edge and then pick up a bit of pus from the dressing. (3) Transfer to a tube of the clotted blood and distribute in the serum. (4) Incubate at 37° C. for 24 hours. (5) Stir the serum with a platinum loop and prepare a smear; stain by the method of Gram.

5. The presence of extremely small gram-negative bacilli with no capsules and no spores are usually sufficient for diagnosis. They have a tendency to occur in

short chains and in parallel rows. In smears of pus they are often intracellular. They stain more deeply at the poles.

6. If cultures are made use plate of one part of fresh sterile blood with two parts or agar inoculated by the surface streak method. Incubate at 37° C. for 48 hours and examine for very small, transparent, gray, firm and finely granular colonies. Prepare smears and stain by the Gram method.

METHODS FOR THE IDENTIFICATION OF BACTERIUM TULARENSE AND THE LABORATORY DIAGNOSIS OF TULAREMIA

1. *Bacterium tularense (Pasteurella tularensis)* produces a disease of rodents transmissible to man with the production of four chief clinical types of disease:

FIG. 256.—BACTERIUM TULARENSE FROM CULTURE ON GLUCOSE CYSTINE AGAR, SHOWING COCCOID AND BACILLARY FORMS IN THE SAME FIELD

Army Medical Museum. Courtesy Edward Francis. U.S.P.H.S. Approx. × 5000.

(*a*) ulceroglandular, due to infection of the skin and later of the regional lymphatic glands; (*b*) oculoglandular, due to infection of the conjunctivae and later of the

lymphatic glands: (c) glandular, with no primary lesion; and (d) typhoid, with no primary or glandular lesions.

2. If secretions are available prepare smears on slides and stain by the Gram method. It is also advisable to make a dark-field examination for *Spirochaeta pallida* to rule out the possibility of syphilis.

3. Inoculate tubes or plates of glucose-cystine agar or blood-cystine agar by rubbing secretion, pus, blood, or better still. a piece of infected tissue over the surface of the medium. Make a blood culture.

4. Incubate at 37° C. for 3 to 5 days and examine for minute, buttery, smooth, easily emulsified, grayish-white colonies. If negative, incubate and study for at least 2 or 3 weeks longer. Prepare smears and stain by Gram method.

5. The bacilli are small, gram-negative, nonmotile, nonspore forming and usually occur singly (Fig. 256). They are pleomorphic: bacillary, coccoid and frequently bipolar. A clear area resembling a capsule often surrounds the bacilli in animal lesions or when mixed with serum. Carbolfuchsin and gentian violet are the best stains.

6. The bacterium produces acid but no gas with glucose, levulose, mannose and glycerin.

Animal Inoculation Test.—1. Inject a guinea-pig subcutaneously with material from glands. ulcers or blood. 2. Shave or pluck the abdominal skin of a second animal; produce and inoculate abrasions. 3. As a general rule both animals will die in 5 to 10 days if *B. tularense* is present with hemorrhagic edema but no pus at the site of inoculation; the lymphatic glands are enlarged with dry, yellowish. caseous material: the spleen is enlarged and dark in color; the liver contains many discrete, white. caseous granules. Prepare smears and cultures.

METHODS FOR THE IDENTIFICATION OF B. PESTIS AND THE LABORATORY DIAGNOSIS OF PLAGUE

1. *B. pestis* (*Pasteurella pestis*) produces a disease of rodents transmissible to man. In the latter two varieties occur: (a) *bubonic plague* due to infection of lymphatic glands and (b) *pneumonic plague* due to infection of the lungs.

2. In the bubonic type prepare smears and cultures of pus aspirated or taken from the glands. If these are small, hard and difficult to aspirate, excise a gland. In the pneumonic type prepare smears and cultures of the sputum. A blood culture is advisable in all cases. From cadavers secure material from glands, spleen and lung.

3. Stain smears by the method of Gram; also with methylene blue or dilute carbolfuchsin.

4. Inoculate plates of blood agar. glycerol agar or 3% sodium chloride agar by the surface streak method. For blood cultures use nutrient broth.

5. Incubate at 30° to 35° C. for 48 hours and examine.

6. The bacilli are short and thick with rounded ends and convex sides, occurring singly or in pairs and at times in short chains or small groups (Fig. 257). They are highly pleomorphic with marked variations in size and staining. They are

gram-negative and bipolar; nonmotile; nonsporulating. In the tissues and in serum broth, capsules are sometimes present.

7. The colonies are small, round, glistening, transparent, colorless, purely granular and entire or with slightly undulating edges. No hemolysis on blood agar. No liquefaction of Löffler's blood serum medium.

8. In broth there is moderate growth with a delicate pellicle from which thread-like growths (stalactites) hang down in the medium.

9. Produces acid but no gas in media carrying dextrose, maltose, mannitol and salicin. Litmus milk is acidulated but not coagulated. Gelatin is not liquefied.

10. Conduct agglutination test with high-titer serum and an antigen of bacilli for confirmation.
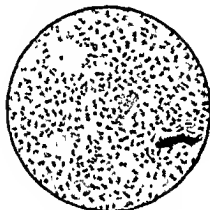
FIG. 257.—BACILLUS PESTIS
(After Mallory and Wright.)
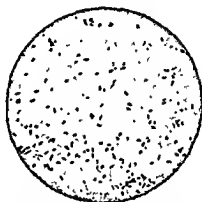
FIG. 258.—BACILLUS PYOCYANEUS
(From Park, Williams and Krumwiede, *Pathogenic Microörganisms*, Lea and Febiger.)

Animal Inoculation Test.—1. Use guinea-pigs or mice.

2. Several hours before inoculation, dip the animals in an antiseptic solution to kill all ectoparasites and then place them in greased glass jars covered with fine mesh top to prevent the escape of any infected parasites.

3. Inoculate subcutaneously with small amounts of original material or with a few loopsful of suspected culture.

4. If *B. pestis* is present, the animals will die in 2 to 4 days with the characteristic lesions in the spleen, liver, etc.

5. Prepare smears and cultures.

Diagnosis of Plague in Rodents.—1. If to be sent to a distant laboratory place the specimen in a tightly sealed container, which is packed in a second container to avoid breakage and ship by express as federal laws prohibit shipment by mail. Decomposition may be avoided by surrounding the specimen container with solid carbon dioxide (dry ice).

2. Wear rubber gloves and long sleeved gowns.

PLATE IV
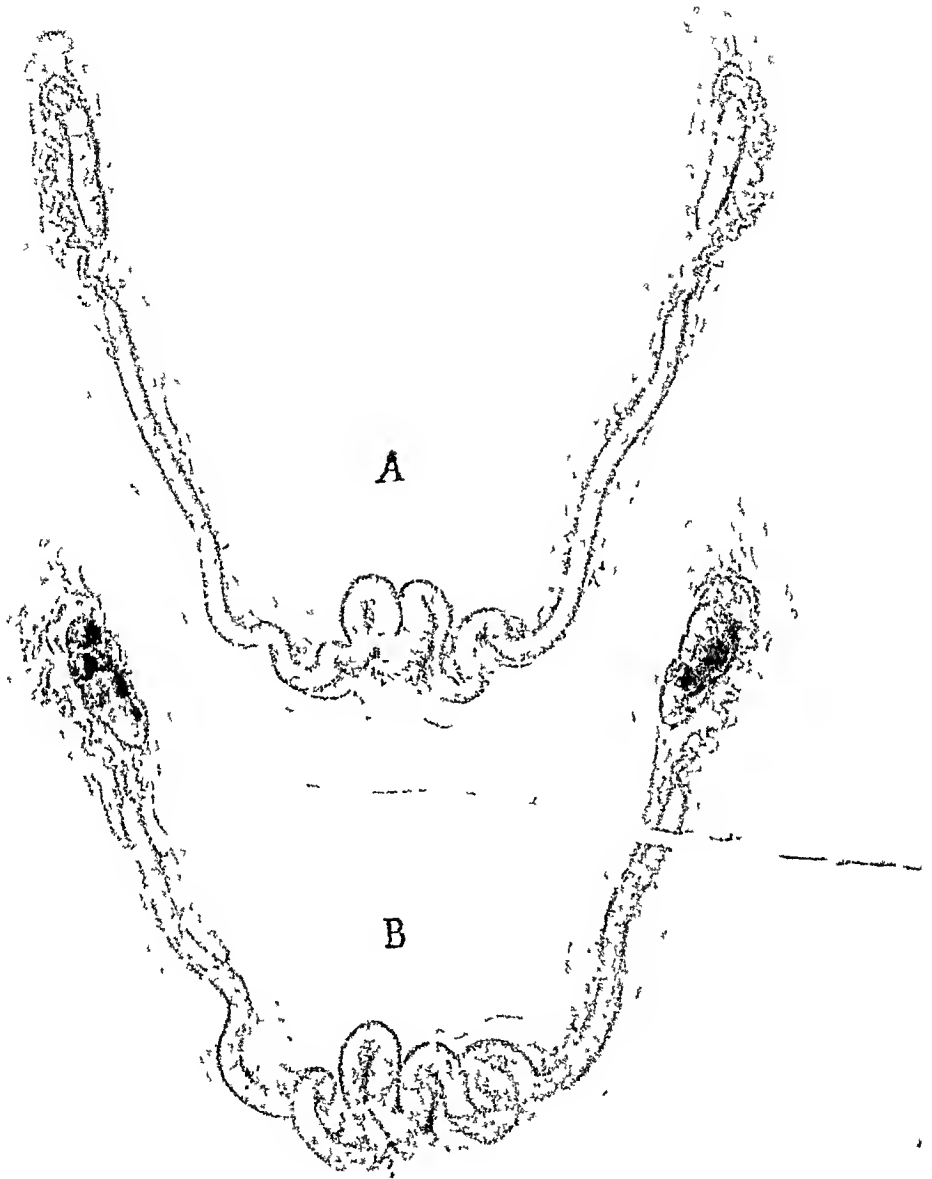
A HORMONE TEST FOR THE DIAGNOSIS OF EARLY PREGNANCY.

A. Bicornate uterus, tubes, and ovaries of a fourteen-week rabbit, thirty hours after injection of 7 c.c. of urine from a non-pregnant patient. This demonstrates a negative result, with no changes occurring in the ovaries.

B. Bicornate uterus, tubes, and ovaries of a fourteen-week rabbit, thirty hours after injection of 7 c.c. of urine from a pregnant patient. This demonstrates a positive result from an approximate five weeks' pregnancy, showing the presence of numerous corpora lutea and corpora hemorrhagica

(From P. F. Schneider, *Surg*, *Ginec.* & *Obst*, Jan. 1931.)

3. Dip the animal in antiseptic solution to kill fleas and other parasites.

4. Conduct an autopsy for macroscopic lesions, although occasionally the disease may occur without recognizable lesions.

5. Examine lymphatic glands which are enlarged, congested, hemorrhagic and later necrotic.

6. Examine the liver for mottling, punctate hemorrhages and pinpoint yellowish spots of fat necrosis.

7. Examine the spleen for enlargement with small, discreet or confluent granules on the surface.

8. Examine the pleural and peritoneal cavities for excess of fluid.

9. Prepare cultures. Examine sections of tissues stained for the bacilli.

10. Tularemia of rats resembles plague so closely that it is necessary to differentiate culturally. The *Corynebacterium pseudotuberculosis* (*Pasteurella pseudotuberculosis*) also produces similar lesions but differs from *B. pestis* in being motile in young broth cultures at 22° C. and by fermenting saccharose with production of alkalinity in milk. The lesions produced by *Trypanosoma lewisii* can be differentiated by finding this parasite in blood smears.

METHODS FOR THE IDENTIFICATION OF B. PYOCYANEUS

1. *B. pyocyaneus* (*Pseudomonas aeruginosa*) is widely distributed in nature and is ordinarily of low pathogenicity. It is particularly likely to occur as a secondary invader in chronic suppurations and especially those involving bone (chronic otitis media; suppurative mastoiditis; osteomyelitis; infected wounds, sinuses, etc.). It may, however, produce primary infections and especially in poorly nourished children (acute otitis media; diarrhea and gastro-enteritis, etc.). It occasionally produces septicemia.

2. In smears and cultures it occurs as a short, slender (Fig. 258), gram-negative bacillus, ordinarily single but sometimes in chains of 4 to 6 elements and occasionally growing out into long filaments and twisted spirals. Sometimes granules are present and the organism may be mistaken for the diphtheria bacillus which, however, is gram-positive.

3. It stains readily; is noncapsulated; is actively motile and nonsporulating.

4. *B. pyocaneus* grows luxuriantly on ordinary media at 37° C. producing smooth, moist, glistening colonies of buttery consistency which later become large, spreading and grayish with irregular edges. The organism in aerobic cultures produces characteristic pigments which diffuse into the medium. One of these is *fluorescein*, which is greenish-yellow or green, soluble in water but insoluble in chloroform. The second is blue-green or *pyocyanine*, soluble in both water and chloroform. It grows readily in broth and especially on the surface with the formation of a pellicle.

5. The organism is usually readily and easily identified by these characteristics.

6. It rapidly digests Löffler's serum medium and gelatin; produces acid, but no gas, in dextrose; produces no indole; does not reduce nitrates.

METHODS FOR THE IDENTIFICATION OF B. MUCOSUS CAPSULATUS

1. *B. mucosus capsulatus* (*Klebsiella pneumoniae*) or the Friedländer bacillus is usually found in the respiratory tract associated with or producing chronic

Fig. 259.—Bacillus Mucosus-Capsulatus (Zinsser)

sinusitis, otitis media and mastoiditis, bronchitis, bronchiectasis, pneumonia and pleuritis and occasionally *pericarditis, conjunctivitis, meningitis and septicemia*.

Fig. 260.—Bacillus Mucosus Capsulatus

(From W. W. Ford, *Textbook of Bacteriology*.)

2. In smears of pus and cultures it occurs as short, plump bacilli or diplobacilli; singly and occasionally in short chains (Fig. 259).

3. The organism stains readily and is gram-negative; *encapsulated* (Fig. 260); non-motile; nonsporulating.

4. It grows well on ordinary media at 37° C. producing round, grayish-white, opaque, convex, smooth, glistening, *mucoid* colonies with smooth edges. On blood agar there is no hemolysis but the medium is browned.

5. It grows well in broth with a pellicle and after several days a marked viscosity.

6. As a general rule the organism is readily identified by these characteristics. Litmus milk is acidified and sometimes coagulated. Acid and gas are produced in dextrose, levulose, galactose, lactose and sucrose; gelatin is not liquefied.

7. By serological tests the smooth type encapsulated bacilli are divisible into types A, B, C and a heterogeneous **Group X**.

METHODS FOR THE IDENTIFICATION OF THE BACILLUS OF RHINOSCLEROMA

1. The bacillus of rhinoscleroma (*Klebsiella rhinoscleromatis*) causes a chronic granuloma of the nose, mouth, pharynx and larynx. It is rare in the United States.

2. It is secured by cultures of the lesion removed by operation, in broth or on plain or blood agar. It is also found in the encapsulated in sections of tissue (Fig. 261).

3. The bacillus is a plump, short rod, with rounded ends, morphologically and culturally very similar to the *B. mucosus capsulatus* of Friedländer. It is nonmotile, gram-negative and encapsulated.

4. It differs from the Friedländer bacillus in producing no gas in dextrose broth, no acid in lactose broth, and not coagulating milk.

METHODS FOR THE IDENTIFICATION OF B. OZENAE

1. The etiology of ozena or atrophic rhinitis is uncertain. *B. ozena* (*Klebsiella ozoenae*) is of doubtful relation to the disease.

2. The bacillus is quite similar to the Friedländer bacillus in being

Fig. 261.—*Bacillus of rhinoscleroma.* Section of tissue showing it within Mikulicz cells. (Kolmer.)

gram-negative encapsulated rods but cultures on solid media and watery and in gelatin stab cultures *B. ozena* spreads out over the Friedländer bacillus forms the characteristic "nail head."

3. According to Page, freshly isolated strains of *B. ozenae* show delay in the production of gas with saccharose and sorbose and delay in lactose (8 days).

4. Another bacterium supposed to have an etiological relation is the *bacillus of Perez* (*Cocco-bacillus foetidus ozenae*) which is motile and noncapsulated. It grows well on ordinary media and gelatin, produces indole and a characteristic fetid odor in culture.

METHODS FOR THE IDENTIFICATION OF B. MALLEI LABORATORY DIAGNOSIS OF GLANDERS

1. *Bacillus mallei* (*Pfeifferella mallei*), which is the cause of glanders in horses and mules and occasionally in other lower animals, sometimes produces disease in human beings whose occupation brings them in contact with

2. Infection takes place by entrance through the broken skin and the mucosa of the mouth or nose. In man infection usually occurs through the skin.

3. Bacteriological diagnosis is usually difficult. Pus from the skin lesions may be used but better results are secured with freshly excised lesions of the skin or portions of submaxillary lymph glands. These should be finely divided with sand

FIG. 262.—GLANDERS BACILLUS
(Zinsser)

in a sterile mortar and planted on plates of 3% glycerol-agar (pH 7.6), glycerol-potato medium, and in 3% glycerol-broth (pH 7.6).

4. Direct smears may be prepared and stained with carbolfuchsin or Löffler's methylene blue but are hardly worth while since the bacillus is not readily recognized by morphological characteristics alone when mixed with other bacteria.

5. Incubate the cultures for 48 to 72 hours as the bacillus at first may grow rather slowly. On glycerol-agar the colonies are whitish or yellowish and usually round. On potato the growth is more characteristic being yellowish, semitransparent and like drops of honey, gradually becoming brownish or amber colored and tenacious. The medium may become green or greenish-brown.

6. Pick off suspicious colonies and transfer to agar slants. Stain smears by Gram method and with carbolfuchsin.

7. The bacillus is gram-negative, slender, nonmotile, nonsporulating and non-encapsulated (Fig. 262). In cultures it is highly pleomorphic, varying greatly in length and width, often showing irregular bizarre forms, and occasionally long filaments with false branching. They stain rather faintly and are sometimes bipolar.

8. Culture can be definitely identified by agglutination test with immune serum, although freshly isolated strains are not easily agglutinated. In conducting this test, heat several 48-hour glycerin-agar slant cultures at 60° C. for two hours and suspend the growths in saline solution carrying 0.3% tricresol. Shake well for a homogeneous suspension and filter if necessary through soft paper.

Set up agglutination tests with 0.5 c.c. of varying dilutions of a known immune serum and add 0.5 c.c. of the bacterial suspension. Include saline controls and preferably a set of controls employing normal serum.

Incubate at 37° C. for 24 hours and read the reactions.

Inoculation of Animals (Straus Test).—1. Inject intraperitoneally a male guinea-pig with a small amount of suspected material.

2. If *B. mallei* is present, an orchitis will develop in 3 to 4 days in about 60
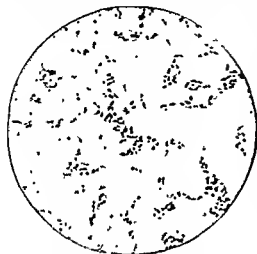
to 70% of cases; in acute glanders positive results are much more likely than in chronic glanders since the bacilli may become avirulent. Similar lesions may be produced by *C. pseudotuberculosis, L. Whitmori* and even by tuberculosis or diphtheria bacilli.

3. As soon as the orchitis is well developed, destroy the animal and culture the testes as just described. If the animal is allowed to live, the testes will abscess and discharge. Lesions will also develop in the liver, spleen, pancreas, lungs, etc.

METHODS FOR THE IDENTIFICATION OF BRUCELLA MELITENSIS, BRUCELLA ABORTUS, BRUCELLA SUIS AND THE LABORATORY DIAGNOSIS OF UNDULANT FEVER

1. *Brucella melitensis* infects goats; *Brucella abortus* infects cows and *Brucella suis* infects hogs. Man may become infected through the ingestion of milk from infected goats and cows or by the flesh of these animals.

2. The three organisms are closely related. In man, laboratory diagnosis is attempted by cultures of the blood, urine and feces and by agglutination and complement fixation tests with serum.

3. Blood cultures are prepared with 10 c.c. or more of blood in 250 to 500 c.c. of infusion broth or liver infusion broth. Incubate at 37° C. and observe for at least 3 weeks, making frequent transfers to duplicate sets of plates of glycerin-agar, "chocolate" blood agar and liver infusion agar. Incubate one set of plates in a jar with 10% CO_2. Examine the plates daily. *Br. abortus* grows fairly well in CO_2 while *Br. melitensis* and *Br. suis* grow very poorly.

4. Collect urine aseptically by catheterization and centrifuge. Inoculate the 3 media with sediment by the surface streak method and incubate as above. The milk of goats and cows may be cultured in the same manner using both the cream and sediment.

5. The colonies are small, round, convex, smooth, glistening and almost colorless. Prepare smears and stain by Gram method.

6. The organisms occur as short straight rods (almost coccal), singly, in pairs end to end, in small groups and occasionally in short chains. They are gramnegative and may show polar staining. They are noncapsulated and nonmotile.

7. Transfer colonies to "chocolate" blood agar. Incubate at 37° C. and examine for purity. Identify the species by the method of Huddleson: prepare plates of beef liver infusion agar (pH 6.6) with (1) 1:50.000 thionin; (2) 1:25.000 basic fuchsin and (3) 1:100,000 pyronin. Inoculate by the surface streak method and incubate 24 to 48 hours; examine:

TABLE V

Species	Thionin	Basic Fuchsin	Pyronin
Br. melitensis	+++	+++	+
Br. abortus	—	+++	+++
Br. suis	+++	—	—

8. *Test for the fermentation of dextrose.* *Br. melitensis* and *Br. suis* produce more acid than *Br. abortus.*

9. In a medium containing organic sulphur, *Br. abortus* and *Br. suis* liberate hydrogen sulphide while *Br. melitensis* does not.

10. Final identification may require macroscopic agglutination tests with antisera for *Br. melitensis*, *Br. abortus* and *Br. suis*; the final dilutions to employ depends upon the titers of the respective sera. Owing to the difficulty of bacteriological diagnosis, agglutination and complement fixation tests with patient's serum are preferred. The methods are described in Chapters XXVII and XXIX. When agglutination tests are conducted, it is advisable to conduct duplicate tests with *B. typhosus* and *B. tularense* at the same time.

Animal Inoculation Test.—The organisms may be isolated from infected tissues, blood, urine or milk by inoculating guinea-pigs subcutaneously or intraperitoneally. If pure cultures are used inject 1 c.c. of a broth culture. If the animals do not die, kill them at the end of seven weeks and examine the lymphatic glands, liver and spleen for small grayish lesions resembling those of tuberculosis. Prepare smears and stain by Gram and by the Ziehl-Neelsen methods. Prepare cultures as described above, incubating one set aerobically and the other with 10% CO_2. Isolate and identify as described above. *Br. melitensis* and *Br. suis* are more infective for guinea-pigs than *Br. abortus.*

The Opsono-Phagocytic Test.—Marked phagocytosis of *Brucella* in vitro by the polymorphonuclear leukocytes in whole citrated blood is regarded as evidence of immunity and an indication of recovery. Slight or no phagocytosis is indicative of susceptibility. The technic after that of Keller, Pharris, Crit and Gaub (Jour. A.M.A., 1936, 107, 1369) is as follows:

1. In a test tube place 0.2 c.c. of 20% sodium citrate solution in physiological saline solution and add 5 c.c. of blood from a vein. Mix. This gives a final dilution of 0.8% citrate which prevents coagulation and inhibits normal opsonins. Use within 6 hours.

2. Shake thoroughly and place 0.1 c.c. in a small test tube; add 0.1 c.c. of a freshly prepared *heavy* suspension of living Brucella from a 24-hour agar culture in saline solution (pH 7.0). The test may be conducted with any of the three types of Brucella but the strain employed should be one known to be susceptible to phagocytosis.

3. Mix and incubate in a water bath at 37° C. for 30 minutes.

4. Withdraw a small amount of sedimented cells with a capillary pipet and prepare smears on slides. Dry rapidly in the air.

5. Cover with 0.5 c.c. of Hasting's stain for 15 seconds to fix and add 1 c.c. of distilled water. Allow to stain for 10 minutes.

6. Wash in water and dry. Examine with oil immersion objective.

7. Count the total organisms in 25 polymorphonuclear leukocytes in different parts of the field and divide by 25 to determine the average number per cell. Report as follows:

Negative: No phagocytosis
Slightly positive: 1 to 20
Moderately positive: 21 to 40
Strongly positive: 41 or more

8. Agglutination and allergic intracutaneous skin tests with the soluble nucleo-proteins are advisable at the same time:

Agglutination	Skin Reaction	Phagocytosis	Interpretation
Negative	Negative	0 to 20	Susceptibility
Negative	Positive	0 to 40	Infection
Positive	Positive	0 to 40	Infection
Negative	Positive	60 to 100	Immunity
Positive	Positive	60 to 100	Immunity

METHODS FOR THE IDENTIFICATION OF B. TUBERCULOSIS

1. Methods for the bacteriological diagnosis of tuberculosis of human beings include the examination of sputum, urine, cerebrospinal, pleural and other fluids for *B. tuberculosis* (*Mycobacterium tuberculosis*) by smears, cultures and animal inoculation.

2. The bacilli are characteristically *acid-fast* but in cultures nonacid-fast forms may be encountered.

3. Human tubercle bacilli occur as slender, straight or slightly curved rods, singly or in small clumps with the organisms at angles to each other. They may stain evenly or show granular and banded forms (Plate IX, Figs. 1 and 3). They occasionally occur as threads and show branching. They are nonmotile, nonsporulating, nonencapsulated and are gram-positive.

4. Bovine tubercle bacilli (Plate IX, Fig. 1) are shorter and more plump and very short forms may be intermixed with somewhat larger forms. They stain irregularly but are, likewise, acid-fast. They are less easily cultivated and are characterized by being more pathogenic for rabbits than human bacilli.

5. The tubercle bacillus grows slowly, the rate depending upon the medium employed. The colonies are ordinarily small, crumb-like, irregular, moist and later dry, yellowish-brown and with a characteristic odor. The bacilli are highly aerobic and in glycerol-broth tend to grow on the surface as a wrinkled pellicle with no turbidity and a slight granular sediment.

Detection of Tubercle Bacilli in Sputum by Smear Examination.—1. Pour the sputum into a Petri dish and pick up with sterilized platinum wire small white or yellow caseous particles: if none is present, choose for examination some of the thicker yellowish or greenish portions.

2. Make at least two smears on glass slides. They should be thin and uniform; never heavy and unevenly distributed. Material may be put on the upper half of a slide and squeezed out with another slide, continuing the rubbing until the sputum is evenly distributed when the slides are separated.

3. Stain with Ziehl-Neelsen's carbolfuchsin for acid-fast organisms.

4. The tubercle bacilli will appear as red, solid or vacuolated, straight or slightly curved rods; other bacteria and cells are stained blue. At least two smears should be examined before a negative report is given and five minutes or more devoted to the examination of each.

5. The average number of tubercle bacilli per field may be recorded according to the following scheme of Gaffky as modified by L. Brown:

> No. 1, only 1 to 4 in whole preparation.
> No. 2, only 1 bacillus on an average in many fields.
> No. 3, only 1 bacillus on an average in each field.
> No. 4, about 2 to 3 bacilli on an average in each field.
> No. 5, about 4 to 6 bacilli on an average in each field.
> No. 6, about 7 to 12 bacilli on an average in each field.
> No. 7, about 13 to 25 bacilli on an average in each field.
> No. 8, about 50 bacilli on an average in each field.
> No. 9, about 100 or more bacilli on an average in each field.
> No. 10, enormous numbers in each field.

Sources of Error.—1. Scratches in the slides may retain the stain and be mistaken for acid-fast bacilli.

2. Incomplete decolorization.

3. There may be tubercle bacilli in the carbolfuchsin washed off from former specimens if the stain is being repeatedly used.

4. Acid-fast bacilli may be present in stale distilled water used for washing slides; also in vaseline and milk bottles used for collection of specimens.

5. Wood fibers, food particles and crystals may retain the fuchsin and resemble tubercle bacilli, although the latter are usually readily differentiated by careful study of morphology.

Detection of Tubercle Bacilli in Urine by Smear Examination.—1. Twenty-four-hour specimens are preferred, collected in a clean (preferably sterile) bottle.

2. Shake the specimen of urine thoroughly; fill 2 centrifuge tubes, 50 c.c. capacity, and centrifuge (if smaller tubes are used more will have to be used or the centrifuging repeated until sediment from at least 100 c.c. of the urine has been collected).

3. Mix the sediment from both tubes or the sediment from 100 c.c. of urine. (If sediment contains crystals, they should be dissolved by very small amounts of either ammonium hydroxide if acid, or acetic acid if alkaline, diluted with water and centrifuged.)

4. By means of platinum loop, transfer sediment to slides.

5. Spread out in thin films and dry.

6. Fix by gentle heat.

7. Stain for acid-fast bacilli by the Pappenheim or Ziehl-Neelsen method.

8. It may be necessary to add a drop of Mayer's egg albumin to the sediment on the slide, before spreading, to facilitate adherence.

PLATE IX
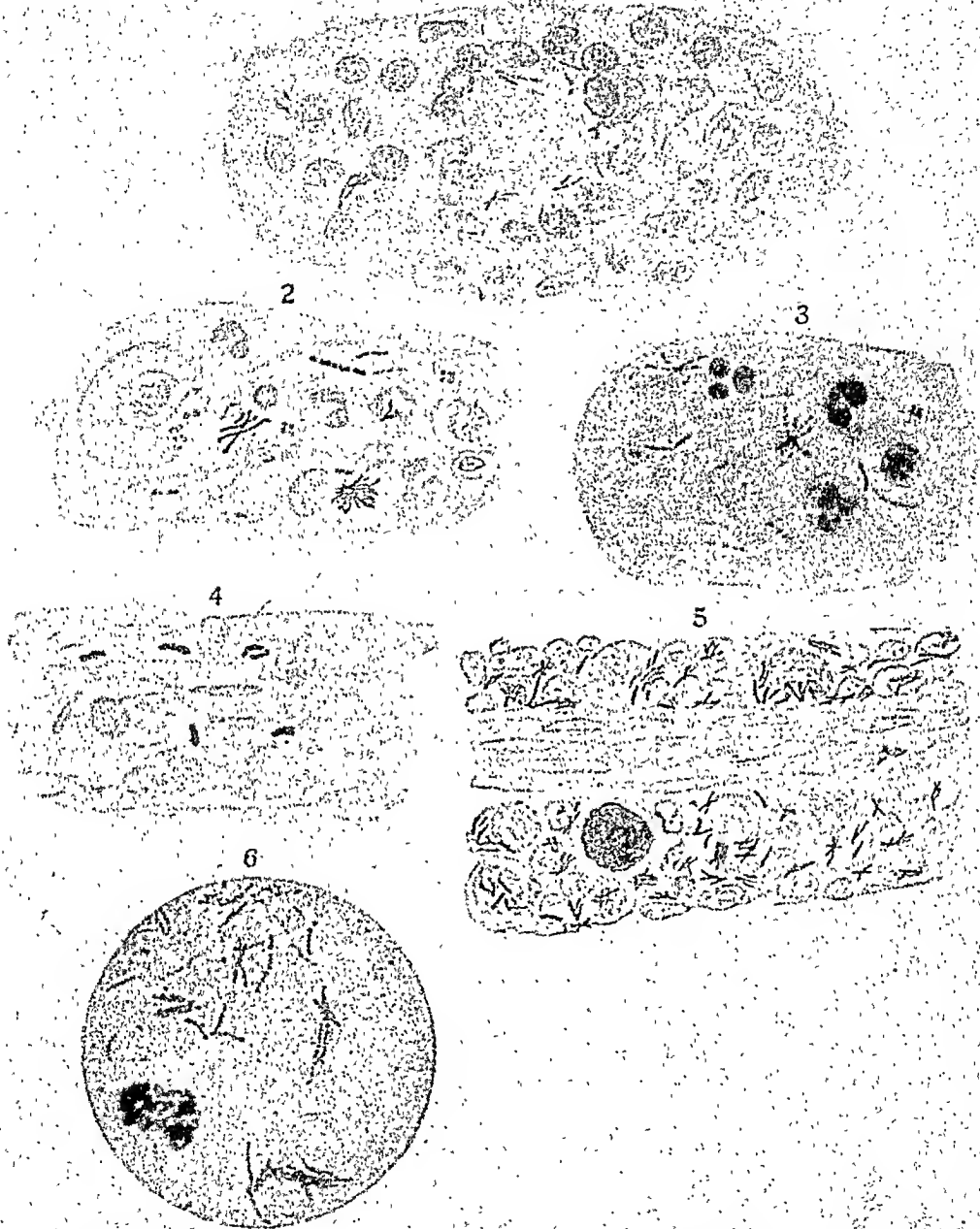
1, Tuberculous lymph node "giant cell" containing tubercle bacilli "human type." Bacilli are red, rest of specimen, blue. Ziel-Neelson stain. × 1000 diameters.

2, Tuberculous sputum from human case. Stain same as above. × 1000 diameters.

3, Tuberculous sputum; human case. Stained by Herrmann's method. Tubercle bacilli are violet, rest of specimen, brown.

4, Pus from tuberculous abscess in cow, "Bovine type" of bacillus. Stained same as 1 and 2. × 1000 diameters.

5, Section through leprous skin showing bacilli in clumps in and out of cells, and large "leprous cell" containing a ball of bacilli. Stained by Ziehl-Neelson method.

6, Photograph of human type of tubercle bacilli from sputum. Bacilli in red, rest of specimen blue. × 1000 diameters. (Fränkel and Pfeiffer).

(From Park, Williams and Krumwiede, Pathogenic Microörganisms, Lea and Febiger,

9. An important source of error is the presence of smegma bacilli which are regarded as usually decolorized by this method.

Detection of Tubercle Bacilli in Feces by Smear Examination.—1. Make thin smear of feces on slide or coverglass; if blood or mucus is present in the feces, this will be more apt to contain bacilli.

2. Dry in air, fix, and stain for acid-fast bacilli.

3. The concentration methods are a great aid and may be conducted in the same manner as for the examination of sputum.

4. Petroff recommends diluting the stool with 2 volumes of water, stirring and filtering to remove the coarse particles. The liquid stool is then saturated with sodium chloride crystals and allowed to stand at room temperature for several hours. Collect the scum with a sterile spoon and place in a wide-mouthed bottle. Add 2 volumes of normal sodium hydroxide, shake well and incubate at 37° C. for 1 to 2 hours. Centrifuge and decant the supernatant fluid. To the sediment add 3 to 4 drops of normal hydrochloric acid to neutralize to litmus or thymol blue. One part may be smeared on tubes of an inspissated medium (Corper or Loewenstein) for culture, a part used for guinea-pig inoculation and a part for making smears to be stained by the usual methods.

Detection of Tubercle Bacilli in Pleural and Spinal Fluids by Smear Examination.—1. Collect and prepare smears as described above for urine; if a coagulum has formed, remove and tease out on a slide with pins.

2. Stain and examine for acid-fast bacilli. Prolonged search is generally required.

3. Tubercle bacilli are the only acid-fast bacilli encountered in spinal fluids. Cultures of the sediment on Corper's potato or egg-yolk medium are frequently positive when the bacilli are present. Very careful examination of smears should reveal the bacilli in 80 to 100%. But in their absence the following changes, if present, should warrant a tentative diagnosis of tuberculous meningitis (Foord and Forsyth): (a) increased cell count producing a slight ground glass appearance of the fluid; (b) marked preponderance of lymphocytes but a moderate number of polymorphonuclears may show in smear-negative cases; (c) the formation of an inverted pine tree web on standing; (d) a colloidal gold curve showing maximum precipitation in the 6th or 7th tube; (e) sugar moderately or markedly reduced (averaging about 36 mgm. per 100 c.c.); (f) chlorides below 650 mgm. To these criteria may be added a marked increase of protein (strongly positive Pandy reaction).

4. Petroff recommends the following method for *pleural* and *peritoneal* fluids: To approximately 10 c.c. of fluid, add 2 drops of 5% solution of tannic acid. Mix well and centrifuge. Decant the supernatant fluid. Prepare and stain smears of the sediment, portions of which may be also used for preparing cultures and inoculating guinea-pigs.

5. Pleural fluids containing large clots may be examined by separating the clot and adding to it equal volumes each of normal sodium hydroxide and 15% solution of antiformin. Shake and digest at 37° C. for about an hour. Centrifuge and decant

the supernatant fluid. To the sediment add a few drops of normal hydrochloric acid; prepare and stain smears. The clots may also be used for cultures and guinea-pig inoculation.

Detection of Tubercle Bacilli in Milk.—1. Use milk as fresh as possible.

2. Centrifuge 20 c.c. at high speed.

3. Take off the cream on top, dilute 4 c.c. with sterile water and inoculate guinea-pig subcutaneously.

4. Inject 1 to 3 c.c. of sediment into additional pigs. Also prepare and stain smears.

5. Acid-fast bacilli in smears may be "butter bacilli" and also produce local lesions but not generalized infections. To prevent these errors, inoculate tubes of glycerin agar with sediment or cream. "Butter bacilli" develop in a few days at 37° C. and also at room temperature.

6. When ready to examine the pigs, inoculate each with 2 c.c. of old tuberculin late in the day. The following morning the tuberculous animals will be dead or dying; conduct autopsies to confirm the results.

Detection of Tubercle Bacilli by Concentration Methods.—*Antiformin Method.*—This is considered useful when direct smears are negative. Examination of a sample of the entire twenty-four-hour *sputum* is recommended. Antiformin is a proprietary preparation and may be prepared by mixing equal parts of liquor sodae chlorinatae, U. S. P. and a 15% solution of sodium hydroxide. The former is prepared by dissolving 600 grams of sodium carbonate and 400 grams of chlorinated lime in 4,000 c.c. of distilled water, allowing the mixture to settle and then filtering the supernatant fluid. Equal parts are mixed with a 15% solution of sodium hydroxide. Keep in a dark bottle in a cool place.

1. Place equal parts of sputum and 50% antiformin solution in small beaker or prepare the mixture in the sputum container.

2. Incubate at 37° C. for 30 minutes, stirring occasionally to insure complete liquefaction or shake for 30 minutes in a shaking machine.

3. Dilute with 3 volumes of sterile water to reduce specific gravity of the solution.

4. Centrifuge for 10 to 30 minutes; pour off supernatant fluid; fill tube with water; centrifuge; continue until all of the fluid has been centrifuged.

5. To sediment left, after pouring off supernatant fluid, add sterile distilled water, mix well, centrifuge and pour off supernatant fluid.

6. By means of a platinum loop transfer sediment to slides.

7. Make smears and stain for acid-fast bacilli.

8. If the sediment does not adhere to the slide, apply a thin smear of Mayer's albumin or raw egg albumin to the slide (egg white, 1 part; water, 10 parts; and formalin, 1 part) and spread the sediment.

9. The sediment may be used for cultural purposes or for inoculation of guinea-pigs, although many tubercle bacilli may be destroyed.

Petroff's Method.—Instead of using antiformin, Petroff recommends adding to the *sputum* an equal volume of 4% sodium hydroxide. The mixture is kept at

37° C. for 15 to 30 minutes, shaking frequently to insure a uniform mixture. After complete homogenization, it is centrifuged at high speed and the supernatant fluid decanted. To the sediment add 2 or 3 drops of normal hydrochloric acid to make it slightly acid and prepare smears as above.

With *urine* Petroff's method is conducted with 24-hour specimens as follows: acidulate with a few drops of 30% nitric acid. To every 1000 c.c. add 2 c.c. of 5% tannic acid solution. Shake well and place in refrigerator for 24 hours. Decant the supernatant fluid and centrifuge the sediment; decant the supernatant fluid. Treat the sediment with 1 c.c. of normal sodium hydroxide solution, which should completely dissolve it. Warm for 30 minutes at 37° C.; dilute with 3 volumes of sterile water and centrifuge. Decant. prepare and stain smears of the sediment. If cultures are to be made, add 5 volumes of normal sodium hydroxide to the sediment; shake well, incubate for 30 minutes and centrifuge at high speed for 5 to 10 minutes (some bacilli are destroyed). Decant the supernatant fluid, add 2 drops of normal hydrochloric acid, mix well and distribute the sediment on the surface of tubes of medium containing one of the static dyes (Corper or Lowenstein). Paraffin the stoppers and incubate. Tubercle bacilli if present will appear in about 2 to 10 weeks.

Method for the Cultivation of Tubercle Bacilli (Corper and Uyei).—1. Use Corper's glycerol water crystal-violet potato or egg-yolk medium.

2. One c.c. of suspected material is beaten to a homogeneous pulp and introduced into a sterile centrifuge tube of 15 c.c. capacity with 1 c.c. of 6% sulphuric acid (17 c.c. of 96% (specific gravity. 1.84) sulphuric acid in 500 c.c. of distilled water) or, better, 1 c.c. of 5% pure oxalic acid (by weight). After incubation at 37° C. for 30 minutes, neutralize with normal sodium hydroxide, checking the reaction with litmus. Add about 10 c.c. of sterile 0.9% sodium chloride solution and centrifugalize. The residue, after the supernatant fluid has been decanted, is seeded lightly on the surface of 3 to 6 tubes of the medium, the culture tube being capped with tinfoil or a sterile cork after the cotton plug has been lightly impregnated with hot paraffin to prevent drying out of the medium.

3. The culture tubes should be incubated in the dark, with due precaution being taken to avoid drying of the medium or contamination. A luxuriant growth should occur on this medium within from 2 to 6 weeks; but if the culture is negative, the tubes should not be discarded for diagnostic purposes until after 3 months' observation at incubator temperature.

4. A few of the ordinary precautions necessary in growing tubercle bacilli by this method are: (*a*) Avoid changes which may occur in the potatoes before autoclaving by not allowing them to stand too long a time after cutting. (*b*) Avoid drying of the culture medium during the long period of incubation necessary by paraffining the cotton stoppers. (*c*) Keep the culture tubes, while in the incubator, in a dark receptacle like a covered tin can or keep the incubator dark, preferably both. (*d*) Maintain a constant temperature of 37.5° C. (*e*) pH of medium 7.0 with isotonicity. (*f*) Eliminate secondary organisms. (*g*) Avoid inhibitory dyes in the medium.

Tubercle bacilli in exudates or other material contaminated with other organisms may be isolated by injecting the material into a guinea-pig and then obtaining material aseptically from the tuberculous lesions. Such material will contain many more tubercle bacilli and none of the contaminating organisms. Otherwise the tubercle bacilli grow slowly and may be readily overgrown by contaminating organisms.

Methods for the Detection of Tubercle Bacilli in the Blood.—In the Löwenstein method, hemoglobin is first removed but this is not essential. Collect 5 to 10 c.c. of blood in 2 or 3 c.c. of sterile 10% sodium citrate in 50 c.c. centrifuge tubes and mix. Add 30 c.c. of sterile distilled water, shake well to produce complete hemolysis and centrifuge thoroughly. Discard the supernatant fluid, add 30 c.c. of sterile distilled water, mix and centrifuge again very thoroughly. Discard the supernatant fluid and transfer the grayish sediment with a sterile pipet to 2 or 3 tubes of Löwenstein's Congo red egg medium. Seal the tubes with wax and incubate at 37° C. in a horizontal position. Then place the tubes upright and incubate for 4 to 8 weeks. If a growth appears prepare smears and stain for acid-fast bacilli. If there is no visible growth, scrape the surface of the medium into a small amount of distilled water. Centrifuge and examine the sediment.

Mishulow and her colleagues state that whole blood may be used as follows: by means of a sterile pipet add a few drops of citrated blood to plates of Bordet-Gengou and Löwenstein media. Add 1 c.c. of sterile distilled water to each plate to facilitate the spread of the hemolyzed blood and secure even distribution. Incubate at 37° C. for 2 or 3 days until the excess moisture has evaporated. Then seal each plate with tape and incubate for two months. Examine once a week with low power microscope lens. Prepare smears and stain for acid-fast bacilli if suspicious colonies develop.

Detection of Tubercle Bacilli by Animal Inoculation.—1. Morning *sputum* is preferred and inoculated at once or after washing with sterile saline solution. If not fresh and if many other bacteria are present, it may be treated with an equal volume of 6% sulphuric acid and neutralized with about an equal volume of 5% sodium hydroxide to avoid septic infection of the animals. If *urine* is to be examined, collect sediment from 100 c.c. *Feces* should be digested in the same manner as sputum.

2. Weigh 2 guinea-pigs and note their weights. Also note their color or otherwise mark them for further identification.

3. Inoculate each pig subcutaneously with the sediment from 50 c.c. of urine, a bean-sized portion of sputum (about 0.5 gm.) or the washed sediment of feces after treatment with sodium hydroxide and oxalic acid as described above. Do not inject into the mammary glands.

4. Bloch recommends damaging the inguinal lymph glands by squeezing between the finger, and injection of the material into these damaged glands (of questionable value).

5. Examine the animals each week for symptoms of tuberculosis. Weigh them and examine site of inoculation for tubercle or tuberculous ulceration; also super-

ficial lymph glands or enlargements. If the subcutaneous glands are enlarged. obtain pus and examine by smears for acid-fast bacilli since saprophytes are destroyed in a few weeks while tubercle bacilli multiply.

6. If the animals show physical signs of tuberculosis at the end of two weeks. one may be killed and the presence of tuberculosis confirmed, in which case the other animal may be destroyed and examined. If necropsy of first animal fails to reveal tuberculosis, allow the other to live for 4 to 6 weeks. then destroy, examine and report positive or negative findings. Or at the end of two weeks inject one of the animals intracutaneously with 0.1 c.c. of a 5% solution of old tuberculin. If no reaction has supervened within forty-eight hours the test should be repeated two weeks later.

7. Should neither pig show any signs of tuberculosis, one should be allowed to live 4 to 6 weeks before destroying and the other for a longer period up to at least 3 months. It sometimes happens that specimens of urine contain organisms other than tubercle bacilli in large numbers which cause the death of the inoculated animals in a few days and require a repetition of the test.

8. Enlarged glands or tissue from other organs (spleen) may be examined for tubercle bacilli by making smears and staining. If acid-fast bacilli are not found, the tissues should be examined histologically for tuberculosis before a negative report is warranted. Smears made from the site of inoculation are especially important.

9. Never base a negative report alone on the absence of enlarged external glands; the internal glands and spleen require examination.

10. Guinea-pigs kept in cages in a room free from tuberculous animals and cared for by healthy caretakers rarely develop spontaneous tuberculosis.

11. While cultural methods are equally valuable, the guinea-pig inoculation test when properly conducted is an excellent method for proving the presence of virulent tubercle bacilli in material that is either known or suspected of containing acid-fast bacilli.

METHODS FOR THE IDENTIFICATION OF B. SMEGMATIS

1. This organism is nonpathogenic saprophyte which may occur in the smegma about the genitalia of both sexes and since it is also acid-fast, may be encountered in urine and thus mistaken for *B. tuberculosis.*

2. It is similar in morphology to the tubercle bacillus but more pleomorphic, sometimes occurring in short comma forms and occasionally as spirals.

3. It is at times cultivated with great difficulty, the media requiring enrichment with serum or hydrocele fluid.

4. For differentiation from the tubercle bacillus, smears should be stained by the Pappenheim method. According to Cole, smegma bacilla may resist decolorization for 4 hours at most, while tubercle bacilli will retain the stain for as long as 24 hours, although this differentiation is not absolutely dependable.

5. The final and more conclusive test for differentiation is by guinea-pig inoculation since *B. smegmatis* is nonpathogenic; or by the cultural method with 6%

sulphuric or 5% oxalic acid which destroy saprophytic acid-fast bacilli but not tubercle bacilli.

6. While in the great majority of instances acid-fast bacilli in urine are tubercle bacilli, yet smears stained by the Pappenheim method should be used and all specimens showing acid-fast bacilli subjected to the inoculation test.

METHODS FOR THE DETECTION OF B. LEPRAE

1. Leprosy is caused by *B. leprae* (*Mycobacterium leprae*) and is detected by the examination of smears or sections of nodules stained by acid-fast technic. Neither cultures nor animal inoculation are of any aid in laboratory diagnosis.

2. Since the initial lesion is often an ulcer of the mucosa of the nose, prepare smears and stain by the method of Ziehl-Neelsen. If desirable, the patient may be given 60 grains of potassium iodide beforehand to produce coryza and increase the nasal secretions.

3. Prepare smears of a skin lesion with a safety razor blade or scalpel and stain by method of Ziehl-Neelsen.

4. If possible remove a portion of lesion by biopsy and place in 4% formalin. Prepare paraffin sections and stain for acid-fast bacilli.

5. *B. Leprae* are acid-fast (Plate IX) and gram-positive. They are rather long, slender and usually straight with pointed ends. Decolorization should not be carried too far as they are more easily decolorized than *B. tuberculosis*.

6. In nasal smears the bacilli are apt to be packed in cells (lepra cells) while in sections of nodules they are found chiefly in the skin, packed in characteristic lepra cells (foam cells) and in the endothelium lining the lymphatics.

7. Lepra bacilli are rarely demonstrable in the anaesthetic type of nerve leprosy.

METHODS FOR THE IDENTIFICATION OF BACTERIUM COLI

1. *Bacterium coli* (*Escherichia coli*) is a normal inhabitant of the intestinal tract of man and all vertebrates. At least 15 species have been identified in feces and soil, some of which have been found in milk and cheese. Only 2 are of special interest in human beings, namely, *B. coli communis* and *B. coli communior*.

2. So many occur in the feces that there is no difficulty in isolation although identification requires special methods for differentiation from other gram-negative, motile bacilli of similar morphology.

3. The material usually submitted for examination for *B. coli* comprise the following: (*a*) *Urine* from cases of cystis and pyelitis; (*b*) *bile* from cases of suspected biliary tract disease; (*c*) *peritoneal* exudates in peritonitis; (*d*) *pus* from wounds, abscesses, the prostate gland and fistulae, especially in the region of the rectum, urethra and kidneys; (*e*) *blood cultures* in suspected septicemia and, (*f*) occasionally *cerebrospinal fluid* in suspected meningitis. The bacteriological examination of water includes tests for *B. coli* from the standpoint of possible fecal contamination; special methods are employed as described in Chapter XXIV.

4. The bacterium grows readily in ordinary media so that cultures of urine, bile, peritoneal exudates and pus of other sources, blood cultures, etc., on plain or blood

agar produce circular, raised, low convex, smooth. white to yellowish, finely granular, moist, buttery colonies which are not adherent. easily emulsified and with entire or undulating edges. It is better. however, to prepare surface streak plates, on the Endo medium on which the colonies of B. coli are pink to red with a metallic sheen. Or the material may be streaked on plates of eosin methylene-blue agar on which B. coli produces large discrete colonies with large, dark, almost black centers. with a greenish metallic sheen. The use of either of these special media immediately facilitates the identification of the organism.

5. Direct smears of such materials and of colonies stained by the Gram method

Fig. 263.—Bacillus Coli Communis (Zinsser)

show short. gram-negative rods (Fig. 263) with no spores and no capsules. Their morphology, however, is not characteristic so that it is impossible to identify and differentiate B. coli from other bacilli of the typhoid-colon group by morphology alone. B. coli is motile.

6. Transplant suspicious colonies to the slopes and butts of tubes of Russell's double sugar agar. After incubation at 37° C. for 24 to 48 hours, B. coli produces acid on the slopes with acid and gas in the butts.

7. For final identification inoculate litmus milk in which B. coli produces acid; peptone water in which indole is produced and fermentation tubes of dextrose and saccharose broths. B. coli communis produces acid and gas in dextrose while B. coli communior produces acid and gas in both dextrose and saccharose. B. coli gives a negative Voges-Proskauer reaction.

METHODS FOR THE IDENTIFICATION OF B. PROTEUS-VULGARIS

1. B. proteus-vulgaris is commonly found in putrefying animal and vegetable materials as well as in the soil and feces. Its primary pathogenicity is slight but it may be a source of important secondary infection in gunshot and other wounds, cystitis, pyelitis and of other mucous membranes as well as possibly producing a type of food poisoning.

2. Materials submitted for its examination are usually feces, urine and pus which may be plated in the same manner as described for the typhoid bacillus.

3. The organism occurs as a straight or slightly curved rod with rounded ends varying greatly in size and shape, singly, in pairs or in chains.

4. Most strains are actively motile; all are noncapsulated, nonsporulating and gram-negative.

5. It grows rapidly on plain agar at 37° C. with the production of thin, bluish-gray colonies that spread rapidly over the entire plate designated by Weil and Felix as H ("Hauch" = film) colonies. Nonmotile variants grow in denser, round colonies designated as O ("Ohne Hauch" = without film). Similar colonies occur in blood agar with zones of brownish hemolysis. On eosin-methylene blue agar the colonies are similar with less tendency to spreading.

6. Pick off suspicious colonies and transplant to slants of plain or blood agar for further identification.

7. The organism digests Löffler's blood serum and gelatin. All strains produce acid and gas in dextrose, galactose and sucrose (may be lost by old strains) and most in lactose and maltose. None ferment salicin, mannitol or mannose. Litmus milk is first rendered slightly acid and then markedly alkaline. Indole is produced by some strains. The Voges-Proskauer test is negative.

8. Cultures of the strain X19 are used in the Weil-Felix agglutination test for typhus fever. It should be of the nonmotile or O dissociative type.

METHODS FOR THE IDENTIFICATION OF B. TYPHOSUS

1. The materials usually examined for *B. typhosus* (*Eberthella typhosus*) are (1) blood cultures; (2) urine; (3) feces; (4) bile and (5) occasionally pus, cerebrospinal fluid, etc.

2. The organism occurs as rods of varying length (Fig. 264), usually singly but sometimes in pairs and short chains; nonsporulating; noncapsulated and highly motile. In broth cultures incubated at room temperature the bacilli are usually long and slender. But it is impossible to identify *B. typhosus* by morphology alone. It stains readily and is gram-negative.

3. The colonies of freshly isolated *B. typhosus* on plain or blood agar are usually round with moderate domes, grayish, transparent to opaque, with entire edges. On special media the colonies vary according to the medium employed.

Blood Cultures.—1. These may be prepared in nutrient broth, Kracke medium or bile broth.

2. Incubate at 37° C. and examine daily. Prepare smears stained by the Gram

method and transfer to plates of eosin-methylene blue agar by the surface streak method.

3. Colonies of *B. typhosus* are translucent, colorless or pinkish.

4. Prepare smears and stain by the Gram method. If gram-negative bacilli are present inoculate the butt and slant of Russell's double sugar agar and incubate for identification as described later.

5. If there is no growth within 10 days the culture may be reported sterile. Positive cultures usually show the organisms within 3 days.

Urine Cultures.—1. It is always advisable to collect *urine* aseptically by catheterization and if *B. typhosus* is present it is usually to be found in pure culture. Urine collected in the ordinary way is almost sure to show *B. coli*, staphylococci and other organisms.

2. With a sterile pipet transfer 2 to 5 c.c. to a flask of nutrient broth. Also inoculate the surface of an eosin-methylene blue plate with 1 or 2 c.c.

FIG. 264.—BACILLUS TYPHOSUS

From twenty-four-hour culture on agar, showing regularity of forms.

3. Incubate for 48 to 72 hours. If there is no growth in this period the specimen may be reported as sterile.

4. If a growth appears examine smears stained by the Gram method. On the eosin methylene-blue medium colonies of *B. typhosus* are translucent, colorless or pinkish. Subculture on slants of Russell's double sugar agar and conduct further tests for identification as described later.

5. Another excellent method recommended by Leifson and particularly useful if the urine is likely to be contaminated with *B. coli* and other organisms is as follows:

To 10 c.c. of urine in a sterile test tube add the contents of one capsule (0.23 grams) of selemite-F enrichment medium (Baltimore Biological Laboratory) and incubate for 24 hours. The selemite inhibits the growth of *B. coli* during this interval but allows typhoid bacilli to proliferate.

Incubate for 18 to 24 hours and then streak a loopful on a plate of desoxycholate-citrate agar and 2 loopsful on a second plate. Incubate 24 hours. Colonies of typhoid bacilli are fairly large, translucent and bluish with slightly granular structure. *B. coli* is considerably inhibited but if colonies develop, they occur large and of even red color.

Bile Cultures.—1. Bile may be aspirated from the gallbladder or collected by the method of Lyon as described in Chapter IX.

2. With a sterile pipet transfer 1 to 3 c.c. to a flask of nutrient broth and incubate at 37° C.

3. Examine daily and if there is no growth over a period of 5 to 7 days the specimen may be reported as sterile.

4. If a growth appears examine smears stained by the method of Gram. Also inoculate plates of eosin-methylene blue agar and tubes of Russell's double sugar agar. For the latter use a long platinum needle, which is first plunged into the butt of the medium and then lightly drawn over the surface of the slant. Conduct further tests for identification as described later.

Methods for the Isolation of B. Typhosus from Feces.—1. Various special media are employed to inhibit the growth of fecal organisms and especially *B. coli* while permitting *B. typhosus* to survive and proliferate in more or less characteristic colonies. The feces should be fresh, as standing for as long as 12 hours may diminish positive findings by 50 per cent.

2. One of the best of these is the *bismuth sulphite agar* of Wilson and Blair. The medium does not keep well in plates even in the refrigerator and poured plates should not be more than 3 or 4 days old when used.

As this medium inhibits the growth of most strains of *B. coli* it is possible and advisable to inoculate plates with relatively large amounts of feces.

Streak a large plate with 3 or 4 loops of feces being careful not to tear the surface. Dilute a loopful in about 1 c.c. of sterile water and spread over a second plate. The heavily inoculated plate should show typical colonies from feces containing relatively few typhoid bacilli while the second plate should show discrete characteristic colonies.

Incubate 24 to 48 hours. The typical colonies of *B. typhosus* are rather small, dry, flat and very black with a metallic luster. They are surrounded by blackish smoky halos although these may not be present if the colonies are close together in which case small, light, green colonies are present similar to those of *B. paratyphosus A* (*Salmonella paratyphi*). Colonies of *B. paratyphosus B* (*Salmonella schottmülleri*) and occasional sulphite reducing strains of *B. coli* are essentially similar to those of *B. typhosus*.

Pick off suspicious colonies and transfer to tubes of Russell's double sugar agar for further identification as described later. The inoculation should be made with a long platinum needle, which is first plunged into the butt of the medium and then lightly drawn over the surface of the slant.

Another excellent method recommended by Leifson consists in thoroughly emulsifying one part of feces in 5 parts of selemite F enrichment medium in test tubes. Or prepare a similar emulsion in distilled water and add 1 capsule (0.23 gram) of the medium (Baltimore Biological Laboratory). Incubate 18 to 24 hours and streak 1 loopful on a plate of desoxycholate-citrate agar and 2 loopsful on a second. Incubate 24 hours and study the colonies:

B. typhosus: large, translucent, granular, bluish

B. coli: inhibited or large red colonies

THE COLLOIDAL GOLD TEST, SHOWING THE REACTION WITH THE CEREBROSPINAL FLUID OF PARESIS ("Paretic Curve").
Complete decoloration occurred in the first four tubes, with partial changes in the next four, yielding a 5 5 5 5 4 3 2 1 0 0 curve. (Frazier)
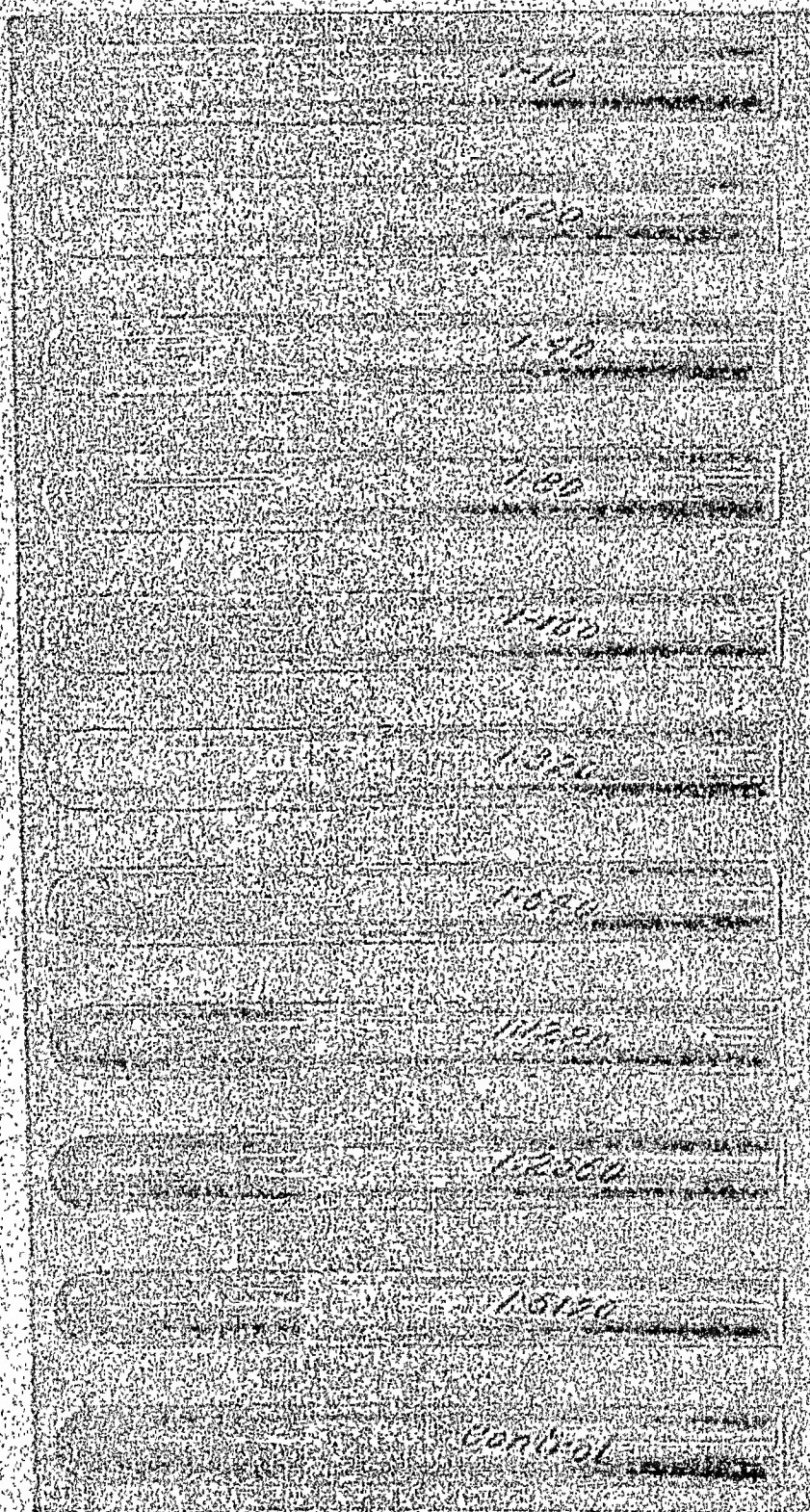
paratyphosus A: large, translucent and somewhat more smooth with more
regular edges than typhoid

faecalis alcaligenes: usually no growth

proteus vulgaris: usually inhibited; colonies with brown centers

pyocyaneus: large, olive colored, slightly granular, opaque colonies

3. Another good medium is *eosin-methylene blue agar* in large plates allowed t
arden and dry by standing for a few hours before use.

Prepare a suspension of about 1 part of feces in 25 parts of sterile saline solutio
nd allow to stand until the large particles have settled.

With a sterile bent glass rod dipped into the emulsion, inoculate a plate b
beginning at the center and passing outward in concentric circles until the entir
surface has been gently smeared. A second plate should be inoculated in the sam
way without redipping.

Incubate at 37° C. for 24 hours. Colonies of *B. typhosus* are usually round wit
high domes, translucent, colorless or pinkish with entire edges.

Pick off suspicious colonies and inoculate the butts and slants of Russell's doubl
sugar agar for further identification as described later.

4. The *Endo* medium may be inoculated in the same manner and incubated a
37° C. for 24 hours. Bacilli of the colon group produce red colonies while typhoid
and paratyphoid bacilli produce bluish-white or colorless colonies. Transplant sus
picious colonies to the butts and slants of Russell's double sugar agar for further
identification as described below.

Methods for Identifying B. Typhosus.—1. Incubate the subcultures on
Russell's double sugar agar at 37° C. for 24 to 48 hours. *B. typhosus* produces acid
but no gas in the butts with the slant unchanged (alkaline).

2. With such results prepare smears and stain by method of Gram. Gram-negative
bacilli are presumably typhoid bacilli but further tests are required.

3. Inoculate fermentation tubes of dextrose, maltose, mannite, saccharose and
lactose broth.

4. Incubate 24 to 48 hours. *B. typhosus* produces acid but no gas with dextrose,
maltose and mannite but not with either saccharose or lactose.

5. Finally *agglutination tests* may be conducted by a *rapid slide* method as
follows:

(*a*) For each colony or slant to be tested, arrange coverglasses and place on
each a small drop of suitable dilution of rabbit-immune serum capable of giving
strong agglutination with *B. typhosus.* As a general rule this may be approximately
1:100.

(*b*) Emulsify in a drop of diluted serum a small portion of the colony to be
studied picked up with a platinum loop. Number the colonies to correspond to the
slides.

(*c*) At the same time prepare controls of each colony in the same manner, using
saline solution instead of serum.

(*d*) Suspend each preparation in hanging drop slides.

(e) Examine microscopically in about 15 minutes. Strong agglutination is presumptive evidence of a colony being typhoid bacilli.

(f) Or place drop of diluted serum on a plain slide and emulsify in each a very small amount of the colonies to be tested. Tilt the slide back and forth for a few seconds over a black background. Agglutination is readily detected by the development of a granular appearance.

If a *test tube method* is preferred it may be conducted as follows:

(a) Arrange 6 small test tubes and place 1 c.c. of distilled water in each.

(b) To No. 1 add 1 c.c. of a 1:25 dilution of typhoid-immune serum and mix well.

(c) Transfer 1 c.c. to No. 2; mix well and transfer 1 c.c. from No. 2 to No. 3 and so on to No. 5, from which 1 c.c. is discarded. The dilutions are 1:50, 1:100, 1,200, 1:400 and 1:800. No. 6 is the control.

(d) To each tube add 1 c.c. of a heavy broth culture of the organism which gives final dilutions of 1:100 to 1:1600.

(e) If the organism is on a solid medium it may be washed off and emulsified in saline solution to give a suspension of approximately 2,000,000,000 per c.c. and used instead of a broth culture. Or the growth may be removed from an agar slant with a platinum loop and emulsified direct in the serum dilution; this, however, gives final dilutions of 1:50 to 1:800.

(f) Mix well and place in a water bath at 50° to 55° C. for 2 hours and then in a refrigerator overnight when the readings are made.

When agglutination tests are indefinite or if cross-agglutination occurs, it is possible to identify the organism by *absorption tests* conducted as follows:

1. Inoculate pint Blake bottles of agar with each culture to be used.

2. After 18 to 24 hours suspend each growth in from 5 to 10 c.c. of saline solution.

3. Centrifugalize each at high speed for 30 to 60 minutes.

4. Remove the supernatant fluids and add 5 to 10 c.c. of 1:50 typhoid or other immune serum to each sediment.

5. Mix well and place in a water bath at 45° C. for three hours, shaking occasionally, and then in the refrigerator overnight.

6. Centrifugalize at high speed for 30 to 60 minutes.

7. Prepare dilutions of 1:100, 1:200, 1:400, 1:800, and 1:1600 of each supernatant fluid and place 0.5 c.c. of each in small test tubes. In tube No. 6 place 0.5 c.c. of saline solution for control.

8. Add 0.5 c.c. of a heavy broth culture or saline suspension of one of the cultures to each tube.

9. Set up duplicate tests with the second or remaining cultures to be tested.

10. Mix well and place in a water bath at 37° C. for 2 hours and in the refrigerator overnight when the readings are made.

11. If a typhoid serum is employed, the cultures completely absorbing the agglutinin (negative reactions) are of typhoid bacilli.

METHODS FOR THE IDENTIFICATION OF PARATYPHOID BACILLI

1. Materials submitted for examination for *B. paratyphosus A* (*Salmonella paratyphi*) and *B. paratyphosus B* (*Salmonella schottmülleri*) are usually feces, urine, blood and bile.

2. The methods of examination are exactly the same as described for the isolation and identification of the typhoid bacillus.

3. The paratyphoid bacilli are gram-negative, motile and very similar morphologically to the typhoid bacillus.

4. On plates of bismuth sulphite agar the colonies of *B. paratyphosus A* are clear and dry or medium-sized and light green with darker centers. Colonies of *B. paratyphosus B* are large and blackish or moist, grayish-brown and confluent. On the Endo medium the colonies of both are like those of *B. typhosus*, being bluish-white or colorless.

5. On Russell's double sugar agar both of the paratyphoid bacilli produce acid with a small amount of gas in the butt but no change (alkaline) on the slants.

6. Both produce acid and gas in fermentation tubes of dextrose and mannite broth. Neither ferments lactose. *B. paratyphosus A* does not ferment xylose while *B. paratyphosus B*. produces acid and gas.

7. *B. paratyphosus A* does not blacken lead acetate agar by the production of H_2S while *B. paratyphosus B* does so.

8. *B. paratyphosus A* produces a persistent slight acidity of milk while *B. paratyphosus B* renders milk strongly alkaline after an initial production of acid.

9. Final identification requires the aid of agglutination and agglutinin absorption tests with the respective immune sera as described above for the identification of the typhoid bacillus. Nearly all strains of *B. paratyphosus A* are agglutinated by antiserum produced by the immunization of an animal with one strain.

METHODS FOR THE IDENTIFICATION OF B. DYSENTERIAE

1. Bacillary dysentery is caused by Shiga's *B. dysenteriae* (*Shigella dysenteriae*) or by the paradysentery bacilli listed below.

2. As the organisms do not produce septicemia, blood cultures are not employed. The material submitted for examination should be *fresh* fecal discharges and preferably the blood-stained mucopurulent portions.

3. Callender states that a presumptive diagnosis can be usually made by the microscopic examination of stained and unstained coverglass preparations of very fresh material for a study of the cells. Blood is present in varying amounts. Polymorphonuclear neutrophils form about 90% of the exudate, many showing nuclear degeneration (ringing) with fat in the cytoplasm. Endothelial macrophages are found containing erythrocytes and leukocytes. They undergo toxic degeneration and form "ghost cells." Plasma cells are abundant early in the disease.

4. The dysentery bacilli occur as rods or coccobacilli and usually singly. They are nonmotile, noncapsulated and nonsporulating. They stain readily and are gram-negative. Since their morphology and staining are not characteristic, the examination of smears of material possess no diagnostic value.

5. Prepare surface streak plates of eosin-methylene blue agar using flecks of blood-stained mucopurulent material. Incubate at 37° C. for 24 hours. Colonies of B. dysenteriae (Shiga) are small, round, translucent, pinkish or colorless. Do not use bismuth sulphite agar as this medium inhibits their growth. Endo medium may be used but since dysentery bacilli do not ferment lactose the colonies are colorless and this medium sometimes inhibits their growth. Blood agar plates may be employed.

6. Transfer suspicious colonies of gram-negative bacilli to slants of blood agar. Incubate 24 hours and examine for motility and by gram stain.

7. For identification inoculate peptone water for indole production, litmus milk and fermentation tubes of dextrose, mannite, lactose and dulcite for acid (gas not produced). The differential characteristics of the important members of the group are shown in Table III.

TABLE VI

Organism	Dextrose	Mannite	Lactose	Dulcite	Indol
B. dysenteriae (Shiga)	+	—	—	—	—
B. ambiguus (Schmitz)	+	—	—	—	+
B. paradys. (Flexner)	+	+	—	—	±
B. paradys. ("y"; Hiss-Park) .	+	+	—	—	+
B. paradys. (Strong).......	+	+	—	+	+
B. alcalescens	+	+	—	+	+
B. dispar (Sonne)....	+	+	+	—	—

8. B. dysenteriae (Shiga) and the other members of the group produce slight acidity in litmus milk followed by neutrality or slight alkalinity except in the case of B. alcalescens, where the medium becomes very alkaline.

9. Final identification usually requires agglutination tests with known anti-dysentery serum. The technic is the same as described under the typhoid bacillus. Polyvalent serum may be employed or monovalent sera for the respective types. The dilutions to employ depend upon the titers of the sera.

METHODS FOR THE IDENTIFICATION OF OTHER GRAM-NEGATIVE NONSPORULATING INTESTINAL BACILLI

1. B. enteritidis (Salmonella enteritidis) is sometimes responsible for food poisoning and gastro-enteritis.

2. B. morgani (Salmonella morganii) is of doubtful pathogenicity but has been found in the diarrheal stools of infants and in the feces of patients with "asylum dysentery."

3. B. suipestifer (Salmonella suipestifer) may produce food poisoning and has also produced other conditions such as septicemia, osteomyelitis, endocarditis and pericarditis.

4. B. aertrycke (Salmonella aertrycke) is pathogenic for rodents but when ingested by man may produce acute gastro-enteritis.

5. *B. faecalis alcaligenes* and *B. lactis aerogenes* are not pathogenic but of interest and importance from the standpoint of being mistaken for pathogens.

6. All occur as gram-negative rods which cannot be differentiated from other members of the colon-typhoid-paratyphoid-dysentery bacilli by morphological characteristics.

7. All grow readily on plates of eosin-methylene blue agar, blood agar and the Endo medium and methods for the isolation of *B. enteritidis*, *B. morgani* (No. 1) and *B. suipestifer* are the same as employed for the isolation of the dysentery and paradysentery bacilli.

8. Identification depends upon the results of sugar fermentation tests, whether or not indole is produced, behavior in lead acetate agar, motility and the Voges-Proskauer reaction. The characteristic reactions are shown in Table IV along with those produced by the more important pathogens.

TABLE VII

Organisms		Dextrose	Mannite	Maltose	Lactose	Xylose	Rhamnose	Saccharose	Dulcite	Lead acetate	Indole	Voges-Proskauer	Motility
Esche-richia-aerobacter group	B. coli communis	⊕	⊖	⊕	⊕	X	X	−	⊕	−	+	−	+
	B. coli communior	⊕	⊕	⊕	⊖	X	X	⊛	⊕	−	+	−	+
	B. acidilactici	⊕	⊕	⊕	⊕	X	X	−	−	−	+	−	−
	B. lactis aerogenes	⊕	⊕	⊕	⊕	X	X	⊕	−	−	−	±	+
	B. cloacae . . .	⊖	⊕	⊕	⊕	⊖	X	⊕	−	−	±	+	±
Salmonella Group	B. paratyph. A ..	⊕	⊕	⊕	−	−	A	−	A	−	−	−	+
	B. paratyph. B...	⊕	⊕	⊕	−	A	A	−	A	+	−	−	+
	B. enteritidis . ..	⊛	⊕	⊕	−	A	A	−	A	A	−	−	+
	B. morgani No. 1 ..	⊕	−	−	−	X	X	−	−	X	+	X	+
	B. abortus-equinus..	⊕	⊕	⊕	−	⊕	−	−	⊕	−	−	−	+
	B. aertrycke .	⊛	⊕	⊕	−	⊕	X	−	⊛	+	−	−	+
	B. pullorum . . .	⊕	⊕	−	−	−	X	−	−	+	−	−	−
	B. gallinarum. .	A	A	A	−	A	X	−	A	+	−	−	−
	B. suipestifer... .	⊕	⊕	⊕	−	⊕	X	−	⊕	±	−	−	+
Shigella group	B. dysenteriae (Shiga)	A	−	−	−	−	−	−	−	−	−	−	−
	B. paradys. (Flexner)	A	A	A	−	−	−	−	−	−	+	−	−
	B. paradys. ("Y").	A	A	−	−	−	−	−	−	−	+	−	−
	B. paradys. (Strong)	A	A	−	−	−	−	−	−	−	+	−	−
	B. typhosus . .	A	A	A	−	A	−	−	−	±	−	−	+
	B. faecalis alcaligenes	−	−	−	−	−	X	−	−	−	+	X	+
	B. proteus-vulgaris	⊕	−	⊖	−	⊖	X	⊛	−	+	±	−	+

⊛ = acid and gas, A = acid, no gas, + = positive, − = negative, X not needed for identification

9. Final identification may require agglutination reactions. Tests conducted by a test tube method as described for the identification of the typhoid bacillus are advised. Immune sera are available for *B. enteritidis* and *B. aertrycke*. Agglutinin absorption tests may be required.

METHODS FOR THE IDENTIFICATION OF THE CHOLERA VIBRIO

1. The cholera vibrio (*Vibrio comma*) occurs in the stools of patients with Asiatic cholera and carriers; also sometimes in the vomitus of this disease as well as in contaminated water, milk or other foods.

2. The "rice water" stools are preferred; do not add glycerin as a preservative. The feces of suspected carriers may be used or swabbings may be taken from the rectum.

3. The organism occurs as slightly curved rods with rounded ends, often resembling a comma (Fig. 265). They occur singly, in S pairs, short chains or spirals. In old cultures they may be small, granular and stain poorly. Involution forms are frequent.

FIG. 265.—CHOLERA SPIRILLUM
(After Frankel and Pfeiffer.)

4. They are very actively motile, noncapsulated and nonsporulating.

5. They stain best with carbolfuchsin and are gram-negative.

6. On plain agar, blood agar or Dieudonnes alkaline blood agar, the colonies are round, low convex, translucent, finely granular ("heaped glass"), buttery and grayish-yellow with entire edges and surrounded by a zone of alpha or beta hemolysis.

7. It rapidly produces indole and reduces nitrates (cholera red reaction), liquefies gelatin, grows abundantly in broth with a thick pellicle being strongly aerobic, reduces nitrates, gives a negative Voges-Proskauer reaction and produces acid but no gas in dextrose, levulose, galactose, maltose, mannitol and sucrose. In litmus milk it produces alkali at the top and acid at the bottom with no coagulation but slow peptonization.

8. Prepare smears of flakes of mucus from a stool and stain by Gram and with carbolfuchsin. If a large number of typical gram-negative, comma-shaped organisms are present, examine a hanging drop preparation. If typical, actively motile vibrios are present, a tentative diagnosis may be made. These procedures are of value in the examination of carriers:

9. Inoculate 2 tubes of alkaline peptone water and prepare plates of Dieudonne's blood agar. Incubate at 37° C.

10. At the end of 8 to 16 hours examine a hanging drop of a peptone water culture and also prepare a smear stained by the Gram method. To 1 tube add 3 to 5 drops of concentrated sulphuric acid as the cholera vibrio gives a pink reaction (cholera red reaction).

11. With the second tube or with suspicious colonies conduct agglutination tests as follows:

(a) Deposit near one end of a slide a drop of agglutinating serum of a dilution of 1:200 (titer not less than 1:4000) and near the other end a drop of saline. Now touch the suspected colony with the point of the inoculating needle, rub up in the drop of saline solution, then flame the point and again *touch* the colony with the point and rub up in the drop of serum solution. Evidence of agglutination will almost instantly be apparent in the latter (if cholera). The drops may be allowed to dry and may be fixed and stained. If agglutination has taken place, it will be evident in the stained specimen to the naked eye, or on slight magnification with the hand lens.

(b) If clumping does not occur, test at least 10 (and preferably 20 or 25) such colonies, and examine the preparation, after standing, with the microscope for vibrios.

(c) If evidence of agglutination on the slide is obtained, or in the event that no agglutination takes place but that the stained preparation shows a vibrio, the colony must be fished and a plain agar slant inoculated for further study.

(d) The crucial test of the specificity of a vibrio is the agglutination test with a serum of high titer.

After incubating the agar slants inoculated with the suspicious colony, or preferably two colonies, for 16 to 24 hours, sufficient culture is on hand for an accurate macroscopic agglutination test in a graded series of serum dilutions.

To be cholera it must agglutinate in a dilution of at least 1:1000 (with a serum having a titer of at least 1:4000) within two hours at 37° C.

If a vibrio is isolated that fails to do this, repeated daily subculture on agar must be made and its agglutinability tested, for observations are on record showing that occasionally (though rarely) a freshly isolated vibrio may show little or no agglutinability but gain it after a series of subcultures.

If no suspicious colonies are found on the plates, make plates from the subcultures which are now 14 hours old. This series of plates is examined, after incubating at least 14 hours, in a manner precisely like that prescribed for the original set.

If under these circumstances no suspicious colonies should be found, the examination must be regarded as negative.

In this case the procedure will have extended over a period of about 36 hours.

Animal Inoculation Test for the Cholera Vibrio.—Emulsify a loopful of culture from an agar slant in 1 c.c. of broth, and inject a guinea-pig intraperitoneally. A fatal peritonitis usually follows within 24 hours.

The Pfeiffer Bacteriolysis Test.—1. To 1 c.c. of 1:1000 high-titer cholera antiserum in broth add a loopful of 18- to 24-hour agar culture and emulsify.

2. Inject into a guinea-pig intraperitoneally.

3. Inject a second animal with a similar emulsion in 1 c.c. of 1:100 normal serum.

4. At intervals of 5, 20, 40 and 60 minutes remove peritoneal exudate with

sharp capillary pipets from each animal and examine microscopically as hanging drop preparations followed by smears stained with carbolfuchsin.

5. A positive reaction is indicated by the vibrios losing motility, swelling and undergoing granular degeneration in the pig inoculated with antiserum.

Method for the Examination of Water for the Cholera Vibrio.—1. Place 100 c.c. in a sterile flask and add 10 c.c. of a sterile 10% solution of peptone in water.

2. Incubate at 37° for 12 hours. Transfer a portion of the surface growth to tubes of alkaline peptone water and prepare plates of plain or Dieudonne's blood agar.

3. If suspicious colonies develop identify by methods described above.

METHODS FOR THE IDENTIFICATION OF LACTOBACILLUS ACIDOPHILUS

1. The *Lactobacillus acidophilus* occurs in the mouth and feces and is believed to be an important factor in the production of dental caries.

2. Materials submitted for examination are usually the feces of individuals taking acidophilus milk and cultures of necrotic teeth.

3. The organism occurs as gram-positive rods, single or in chains and occasionally filamentous.

4. Colonies on tomato juice peptone agar or casein digest agar, are usually large and rough or woolly but may be smooth and round.

5. Litmus milk is coagulated and acidified.

6. Maltose, sucrose, lactose and raffinose are almost always fermented; mannitol rarely and salicin by about one-half of the cultures.

METHODS FOR THE LABORATORY DIAGNOSIS OF HEMORRHAGIC SEPTICEMIA OF THE LOWER ANIMALS

1. *Pasteurella boviseptica (bollingeri)* causes hemorrhagic septicemia in cattle; *Pasteurella equiseptica* in horses; *Pasteurella suilla* in hogs; *Pasteurella oviseptica* in sheep and *Pasteurella avicida* in fowls (fowl cholera).

2. Blood cultures, blood smears on slides, tissues and exudates of edematous swellings, lymphatic glands, spleen and other organs should be examined.

3. Prepare smears and stain with carbolfuchsin. Use Wright's stain for smears of blood, spleen and kidneys.

4. Prepare cultures on plates of blood agar; also on eosin-methylene blue agar for *B. coli* which at times are present and apparently pathogenic.

5. Incubate at 37° C. for 48 hours and examine; prepare smear and stain by Gram method and methylene blue.

6. The organisms occur as small ovoid rods, rounded ends, occurring singly, in pairs or in small bundles.

7. They are nonmotile; noncapsulated in cultures; nonsporulating; gram-negative and bipolar.

8. The colonies are small, round, amorphous, translucent, grayish-yellow to bluish and buttery with no hemolysis but browned.

9. Agglutination tests with specific sera may be employed according to titer although group reactions occur.

10. Inject 1 c.c. of broth culture or tissue emulsion into rabbits intravenously or mice intraperitoneally. If material is grossly contaminated inject subcutaneously. Death usually occurs within 1 to 4 days with slightly swollen spleen, petechial hemorrhages of the serous membranes and laryngotracheitis. Prepare cultures of heart blood, spleen, kidneys, peritoneal fluid and lymphatic glands.

METHODS FOR THE IDENTIFICATION OF THE BACILLUS OF SYMPTOMATIC ANTHRAX (BLACKLEG)

1. The bacillus of symptomatic anthrax (*Clostridium chauvaei*) is pathogenic for cattle, sheep and goats but the disease is usually confined to cattle and is known as "blackleg."

2. Prepare smears of the exudates and stain by the Gram method.

3. Prepare cultures on dextrose blood agar plates and in liver broth. Cultivate anaerobically as the organism is strictly anaerobic. The colonies are circular with a slightly granular compact center, from which a thinner peripheral zone emanates made up of a tangle of fine threads.

4. The bacillus is a large, motile, sporulating rod with rounded ends (Fig. 266). The spores may be located either near the end or centrally and those showing end spores are spoon-shaped. When freshly isolated from tissue (in contradistinction to anthrax) the organisms show the presence of spores.

FIG. 266.—BACILLUS OF SYMPTOMATIC ANTHRAX
(After Zettnow.)

5. The organism ferments dextrose, maltose, lactose and saccharose with the production of acid and gas. Mannite and salicin are not fermented. Indole is not produced.

6. Inject the hind leg of a guinea-pig with 1 c.c. of culture or an extract of tissue. Death usually occurs in 18 to 36 hours with emphysematous gangrene, giving off an odor like that of butyric acid. Examine stained smears for the sporulating bacilli.

METHODS FOR THE LABORATORY DIAGNOSIS OF NECROBACILLOSIS

1. *Actinomyces necrophorus* produces a variety of conditions collectively referred to as "necrobacillosis" including foot rot, necrotic stomatitis of swine, calf diphtheria, liver abscesses in cattle, sheep and hogs, gangrenous dermatitis and

secondarily in the intestinal ulcers of hog cholera and in canker of the foot in the horse. A few human infections have occurred with the production of localized vesicular and gangrenous dermatitis and possible puerperal infections.

2. The organism is a strict anaerobe and very difficult to cultivate. If smears of necrotic material show numerous slender, gram-negative beaded filaments meas-
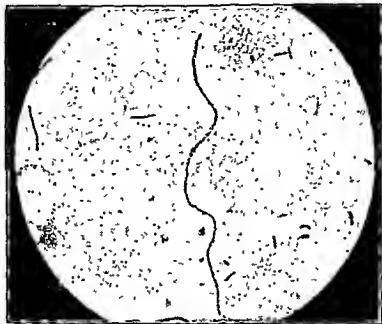
Fig. 267.—Actinomyces Necrophorus
(Courtesy of Dr. Frederick W. Shaw)

uring 100 microns or more in length which fail to grow aerobically, the diagnosis is usually justified (Fig. 267).

3. Inject a rabbit subcutaneously in the median abdominal line with finely divided tissue and pus. A local necrosis is produced from which the organism may be recovered in large numbers.

METHODS FOR THE BACTERIOLOGICAL DIAGNOSIS OF ERYSIPELOID OF MAN AND SWINE ERYSIPELAS

1. Erysipeloid (Rosenbach) of man and "swine erysipelas" are caused by *Erysipelothrix rhusiopathiae* (Kitt).

2. Veterinarians are sometimes infected by handling diseased swine and in the United States the disease is common among handlers of fish (Klauder and Harkins).

3. In human beings the acute septicemic type is rare; infection usually occurs about the fingers and is variously regarded as "fish poisoning" or a pyogenic skin infection. Polyarthritis may occur (Klauder).

4. Excise pieces of skin about 3 millimeters square from infected areas; grind in a sterile mortar and inoculate tubes of hormone broth as the organism grows on ordinary media. Incubate aerobically at 37° C.

5. The organism occurs as a small, slender, gram-positive rod; singly and in chains: sometimes branching: often granular; nonmotile; noncapsulated; nonsporulating. Old cultures are thread-like.

6. On agar the colonies are small, colorless, translucent, moist and homogeneous. Poor growth in broth. Hemolysis on blood agar plates. Ferments dextrose and lactose.

7. Inoculate mice intraperitoneally or pigeons intramuscularly with 0.2 c.c. of 2 to 4 day cultures; usually fatal and pure cultures may be obtained from the blood of the heart by plating on blood agar.

8. Conduct agglutination and complement-fixation tests with swine immune serum. Immune serum also gives a precipitin reaction with filtrates of broth cultures and according to Ascoli, also with extracts of infected tissues.

METHODS OF EXAMINATION IN BACTERIAL FOOD POISONING AND FOR THE IDENTIFICATION OF B. BOTULINUS

1. Food poisoning may be caused by the ingestion of various bacteria or their products but it is frequently difficult and sometimes impossible to determine the exact cause of outbreaks.

2. This type of food poisoning should not be confused with "ptomaine poisoning" due to the ingestion of decomposed foods.

3. While various organisms belonging to the colon, proteus, paradysentery, staphylococcus and streptococcus groups have been found or suspected in different outbreaks, only the following have been definitely incriminated: (a) *Bacillus enteritidis;* (b) *B. aertryke;* (c) *B. paratyphosus A* and *B;* (d) *B. suipestifer;* (e) *B. botulinus* and (f) certain staphylococci, the latter being incriminated in food poisoning from the eating of cream-fillers in pastries and ice cream, chicken salad, etc.

4. In investigating an outbreak (a) secure leftover portions of the suspected food, pack in ice and examine as soon as possible; (b) secure specimens of vomitus and feces for bacteriological examination and at a later date samples of patient's serum for agglutination tests; (c) try to determine the source of the food and secure feces for bacteriological examination and blood for agglutination tests from the handlers and especially cooks suspected as carriers and (d) specimens of blood, spleen, liver and intestines from fatal cases for bacteriological examination.

5. Prepare smears of the foods and stain by Gram to obtain some idea of the predominating organisms.

6. Prepare plates of eosin-methylene blue agar and blood agar for the possible detection of any of the above organisms excepting *B. botulinus.* Incubate for 24 to 48 hours. Study a large number of colonies. Prepare smears and stain by Gram. Subculture on slants of blood agar or Russell's double sugar agar. Staphylococci

and streptococci are easily recognized. Other organisms must be identified according to staining, motility, **fermentation of carbohydrates, indole production,** behavior in litmus milk, liquefaction of gelatin, the Voges-Proskauer reaction agglutination tests, etc.

7. Feces, vomitus, etc., may be plated and examined for the above organisms according to methods given in this chapter for the respective organisms.

8. For *B. botulinus* (*Clostridium botulinum*) anaerobic cultures are required if this organism is suspected. Inoculate 2 tubes of Robertson's medium; heat one of them at 70° C. for 20 minutes and then prepare plates of blood agar by the surface streak method. Incubate both tubes and the plates anaerobically at 35°-37° C. Prepare smears and stain by Gram.

9. *B. botulinus* occurs as a large gram-positive rod with rounded ends, singly or in short chains. Spores are oval, larger than the bacilli, and usually at or near the ends. They form best in sugar-free media at 20° to 25° C.

10. The bacilli are motile and noncapsulated.

11. On blood agar the colonies are irregularly round, umbonate, smooth centers with fimbriate margins and alpha type of hemolysis.

12. In cooked brain broth there is abundant growth with gas and butyric acid odor; the brain is digested and blackened.

13. Type A produces acid and gas in glucose, maltose and salicin; types B and C do not ferment salicin. Types A and B ferment glycerol; type C does not.

14. Test the food for the toxin of *B. botulinus* as follows: (a) Prepare a suspension or dilution of the food (usually the juice in canned vegetables) in sterile saline solution and centrifuge. (b) Give each of 4 guinea-pigs of blood agar 1 c.c. by subcutaneous injection. (c) At the same time inject one intraperitoneally with 1 to 1.5 c.c. of Type A antitoxin; a second with a like amount of Type B antitoxin and a third the same amounts of both the A and B antitoxins. The fourth pig is a control. If the toxin is present the results will be somewhat as follows:

TABLE VIII

Guinea pigs Protected with Antitoxin	With A Toxin	With B Toxin	With A and B
Pig 1 (Type A)	Live	Die	Die
Pig 2 (Type B)	Die	Live	Die
Pig 3 (Types A and B)	Live	Live	Live
Pig 4 (none)	Die	Die	Die

15. A similar test can be made with anaerobic broth cultures.

METHODS FOR THE BACTERIOLOGICAL DIAGNOSIS OF VINCENT'S ANGINA AND FUSOSPIROCHETAI GINGIVITIS

Plaut-Vincent's angina is caused by *Spirocheta vincentii* (*Borrelia vincentii*) in symbiosis with *Bacillus fusiformis*. These organisms also produce a type of gingivitis commonly called "trench mouth" as well as stomatitis and ulcerating lesions in other parts of the body.

Plaut-Vincent's Angina.—1. Smears are required for diagnostic purposes as the organisms are anaerobic and difficult to cultivate. These should be made on glass slides and not too thin. Swabs accompanying cultures on Löffler's blood serum for diphtheria bacilli may be used for preparing smears and this is a good routine practice as Plaut-Vincent's angina may be mistaken clinically for diphtheria.

2. Dry in air.

3. Fix by passing through flame 4 times.

4. Cover with carbolfuchsin diluted 1:10 with water; heat gently and stain for 2 minutes. Stain second slide by method of Gram.

5. Wash in water and dry.

6. Examine with oil-immersion lens for fusiform bacilli and spirilla. The

FIG. 268.—FUSIFORM BACILLI AND SPIRILLA OF VINCENT'S ANGINA (Zinsser)

former are gram-negative (variable), long, slightly curved with pointed ends and showing faintly staining granules. The latter are large, wavy spirals (Fig. 268).

7. Both organisms are also readily seen in wet preparations with high dry or oil-immersion objectives or by dark field examination.

Fusospirochetal and Amebic Gingivitis.—1. Wet and stained preparations are recommended.

2. The material should be collected with care, especially from gingival pockets with suitable instruments or after expression by pressure.

3. Prepare several smears. Dry in the air. Fix with heat. Stain with 1:10 carbolfuchsin for 2 or 3 minutes. Wash with water, dry, and examine with oil-immersion lens for spirochetes (*Spirochaeta microdentium*; *Spirochaeta macrodentium*, etc.).

4. An occasional spirochete is normal. But large numbers and tangled masses represent a pathological increase (spirochetic gingivitis).

5. Fusiform bacilli may also be found in association with spirochetes (*Spirochaeta vincentii*) constituting *spirofusillar gingivitis*, a form of Vincent's angina infection of the gums, or trench mouth.

6. A few spirochetes and fusiform-shaped bacilli resembling *B. fusiformis* are

to be found in most mouths and do not alone constitute evidences of infection; but the presence of large numbers is regarded as pathological.

7. *Leptotrichia buccalis* is frequently found in the mouth and may be mistaken for fusiform bacilli. They occur as long, gram-positive bacilli or filaments. They do not grow under ordinary aerobic conditions.
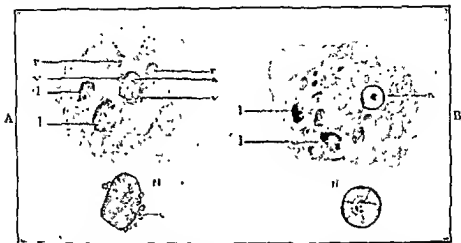
Fig. 269.—Endamoeba Gingivalis

A, trophozoite stained with Wright's stain to show characteristic globules (1) distributed near nucleus in condensed areas of cytoplasm. *B,* a similar form stained by the Heidenhain's iron hematoxylin method which fails to show the perinuclear structures. *N,* nuclear detail at higher magnification. *i,* ingested leukocyte fragments. *n,* nucleus, *r,* ingested red cell fragments. *r,* perinuclear globules or "vacuoles." (Original drawings by Urdle.)

8. Wet preparations are especially desired for examination for *Endamoeba gingivalis* (Gros) which resemble *E. histolytica* (Fig. 252): (Fig. 269).

Warm a slide. Place a drop of warm saline solution. Add a small amount of gingival secretion. Cover with coverglass and examine for motile amebae with high dry lens and with the light well reduced.

Spirochetes may be likewise detected in these preparations or by dark-field examination.

An occasional ameba may be found in the absence of gingivitis. One or more per field, however, represents an increase and may produce gingival infection by opening up avenues of bacterial infection.

METHODS FOR THE LABORATORY DIAGNOSIS OF RECURRENT FEVER

1. *Borrelia recurrentis (Spirochaeta obermeieri)* and *Borrelia novyi (Spirochaeta novyi)* may be found in blood films fixed with methyl alcohol and stained with diluted carbolfuchsin or after staining with Jenner's or Wright's blood stains (Fig. 270). Careful search for the spirochetes is usually required.

2. Cultures are not employed.

3. Inject 0.2 to 0.5 c.c. of patient's blood or clots broken up in sterile saline solution intraperitoneally into a white mouse under aseptic precautions.

4. Examine a drop of blood from the tail on a slide covered with coverglass with high dry or oil-immersion lenses each day over a period of at least 5 to 14 days for spirochetes. Infected mice are likely to survive for months with recurrent spirochetemia.

METHODS FOR THE LABORATORY DIAGNOSIS OF INFECTIOUS JAUNDICE

1. Examine for *Leptospira icterohaemorrhagiae* in the urine and blood.

2. Urine may be centrifuged and the sediment examined by dark-field method.

3. Catheterized urine is suitable for cultures. Inoculate tubes of the rabbit-serum medium with several loopsful of sediment and cultivate anaerobically.

4. Inject white guinea-pigs (8 to 10 ounces) intraperitoneally with sediment suspended in saline solution (same technic as inoculation for tubercle bacilli).
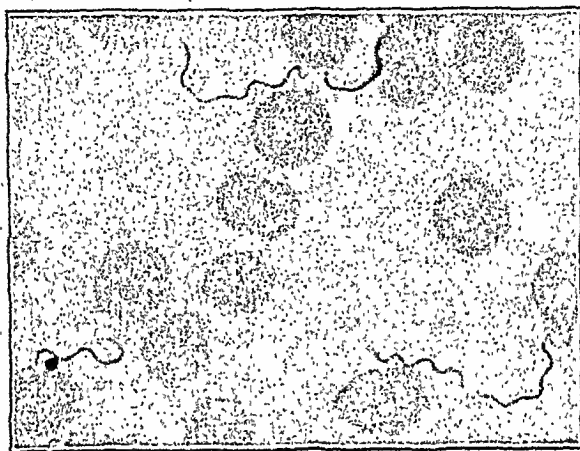
FIG. 270.—BORRELIA RECURRENTS (After Calkins)

5. Inoculate white guinea-pigs of same weight intraperitoneally with 0.5 to 1 c.c. of citrated blood or with clots broken up in sterile saline solution, taken during the first week of the disease.

6. Inoculate rabbit-serum medium with 0.5 c.c. of blood; cultivate anaerobically.

7. Incubate all cultures at 25° C. and examine each weekly by dark-field for at least 4 weeks for motile leptospirae.

8. Examine the guinea-pigs daily for jaundice of the skin and sclerae. Take temperature daily. When a marked rise occurs, remove 1 or 2 c.c. of blood from the heart in an equal amount of 1% solution citrate solution. Examine by dark-field for leptospirae. If organisms are found, chloroform the animal. Look for jaundiced tissues and numerous petechial (butterfly) hemorrhages, especially in the lungs and inguinal region.

9. Prepare sections of the liver and kidneys to be stained for leptospirae.

Agglutination and Lysis.—1. If a culture is available, prepare equal parts of 0.5 c.c. of culture and 1:2, 1:4, 1:8, 1:16, and 1:32 dilutions of patient's serum in small test tubes. Place in water bath for two hours. Examine each and a control for agglutination by dark-field.

2. In a test tube place 0.5 c.c. of culture (upper portion), 0.5 c.c. of the patient's fresh unheated serum and 1.5 c.c. of saline solution. Mix and inject the whole into the peritoneal cavity of guinea-pig. At intervals of 15 minutes withdraw a small amount with fine capillary tubes and examine by dark-field for evidences of agglutination and lysis.

3. Inject a second pig with a control mixture, using normal human serum.

4. If antibodies are present, agglutination with partial or complete lysis usually occurs within an hour.

METHODS FOR THE BACTERIOLOGICAL DIAGNOSIS OF SYPHILIS

1. *Spirochaeta pallida* (*Treponema pallidum*) may be found in primary and secondary lesions by dark-field examination (recommended) or by special staining methods (Fig. 271).

2. Cultures are not employed.

3. Secretions, bits of tissue, spinal fluid and material aspirated from enlarged

Fig. 271.—Spirochaeta Pallida: Levaditi Method (Zinsser)

lymph glands may be inoculated into the testicles of rabbits. Full-grown and healthy animals should be employed. From 0.2 to 1.0 c.c. of fluid or emulsion should be injected into the center of each testicle with a sterile syringe after sterilization of the skin with iodine. Syphilitic orchitis develops in three to six weeks with numerous spirochetes to be seen by dark-field examination. Chancres of the scrotal skin may occur.

METHODS FOR THE BACTERIOLOGICAL DIAGNOSIS OF RAT-BITE FEVER

1. Rat-bite fever is primarily a disease of wild rats transmissible to man, rats and other animals by bites.

2. The causative organism is *Spirochaeta morsus minus* (*Spirillum minus*).

3. Inoculate white mice or guinea-pigs intraperitoneally with the patient's blood (citrated), exudate from the initial lesion, serum expressed from erythematous patches, material aspirated from lymphatic glands, or ground-up tissue. Examine wet preparations of the blood daily for the organism (Fig. 272). If found, prepare mixtures of patient's serum with the blood for loss of motility of the organism. This is a confirmatory test but is often negative, uncertain and subject to error.

4. Examine wet preparations of the patient's blood by dark-field examination although the organism is rarely detected with certainty by this means and there is likelihood of mistaking "artifact spirochetes" for the actual organism. Make

similar examinations of secretions from the initial lesion, which are much more likely to be positive.

5. Smears may be stained with the Wright or Giemsa stains.

6. Cultures are not required.

FIG. 272.—SPIROCHAETA MORSUS MURIS

1, in blood of guinea-pig. Short form. Wright's stain. (×1500.) *2*, in blood of white mouse. Long form. (Army Med. Museum 50281, from Francis.) *3*, in blood plasma of guinea-pig. Flagellum at each end stained by Fontana-Tribondeau silver method. (Army Med. Museum 50417, from Francis.)

METHODS FOR THE LABORATORY DIAGNOSIS OF RABIES

1. Rabies is a disease of dogs, cats, coyotes, wolves and other animals caused by a filtrable virus occurring in the saliva and transmissible to man and other animals through bites and other injuries.

2. Laboratory diagnosis is made by the examination of smears and sections of the hippocampus major (cornu ammonis) and cerebellum for *Negri bodies;* also by the inoculation of rabbits or guinea-pigs with emulsions of brain substance.

3. When removing the brain from an animal wear heavy rubber gloves; it is also advisable to wear goggles. All instruments should be boiled immediately and the table disinfected by wiping with formalin solution.

4. For removing the brain dissect away the skin and muscles and saw through the head longitudinally, thus separating the two hemispheres.

5. The diagnostic Negri bodies can be found in about 96% of instances by preparing simple touch preparations of the hippocampus major, cerebellum and cerebral cortex (near the rolandic fissure) as follows: Press a scrupulously clean slide, free from flaws, several times against the cut surface with sufficient pressure to cause the tissue to spread out over the slide, leaving thin, even films upon the surface.

6. Place slides in absolute alcohol for one to five minutes.

7. Remove from alcohol and allow to dry in air.

8. Cover smears with the following stain and steam gently for 2-3 minutes:

Sat. alc. sol. fuchsin ....5-8 drops
Loeffler's meth. blue .... 15 c.c.
Distilled water sufficient to .... 50 c.c.

9. This stain will keep fairly well in a refrigerator.

10. Wash with water and dry.

11. Locate the ganglion cells with low power objective and then examine the cells with an oil immersion objective for Negri bodies, which appear as round or oval bodies, variable in size, stained magenta and sometimes showing bluish dots or granules (Plate IX). Nerve cells appear light blue; erythrocytes are salmon or yellow.

12. *Mann's method* is also satisfactory as follows: Without drying, fix the smears with methyl alcohol for 2 or 3 minutes. Stain for 5 minutes with the following:

> 1% aqueous solution of Grubler's methylene blue ...... 35 c.c.
> 1% aqueous solution of Grubler's B. A. eosin .... 35 c.c.
> Distilled water .... 100 c.c.

Wash in water and rapidly pass the smears through 50, 75, 95 and two changes of absolute alcohol, clean in a mixture of equal parts of xylol and oil of cloves and examine microscopically. When properly prepared and stained, the nerve cells appear light blue and the Negri bodies pink. Erythrocytes stain an orange color.

13. If direct smears are negative prepare sections as follows: (*a*) Place pieces of tissue not over 1 by 1 by 0.2 centimeter in 10 parts of Zenker's fluid for 8 hours. (*b*) Wash in running water for 8 to 24 hours. (*c*) Place in 80% alcohol for 1 hour and then in 95% and absolute alcohols for 1 hour each. (*d*) Place in xylol for 1 hour and then in xylol-paraffin and finally paraffin in the incubator for 1 hour each. (*e*) Imbed in paraffin and cut thin sections. (*f*) Stain by Mallory's or Goodpasture's methods (Chapter XVIII). *Seller's stain* is quite satisfactory:

SOLUTION A

> Methylene blue .... 15 gm.
> Methyl alcohol .... 100 c.c.

SOLUTION B

> Basic fuchsin .... 32 gm.
> Methyl alcohol .... 100 c.c.

Just before it is to be used, mix 150 c.c. of solution A with 50 c.c. of solution B and 250 c.c. of methyl alcohol. This mixture is not stable and should be used the same day it is made. Test the stain before use on brain tissue preferably on tissue containing Negri bodies. The chromatin should stain blue and cytoplasm red. If a clear-cut differentiation of cytoplasm and chromatin is not obtained additional amounts of either solution A or B is added until the desired effect is obtained.

The following method of making the preparations of the brain is suggested by P. T. Buck. Cut a fairly thin section of the brain to be examined and put on the end of a piece of cork stopper, pressing the edges firmly against the cork. Dip

PLATE X
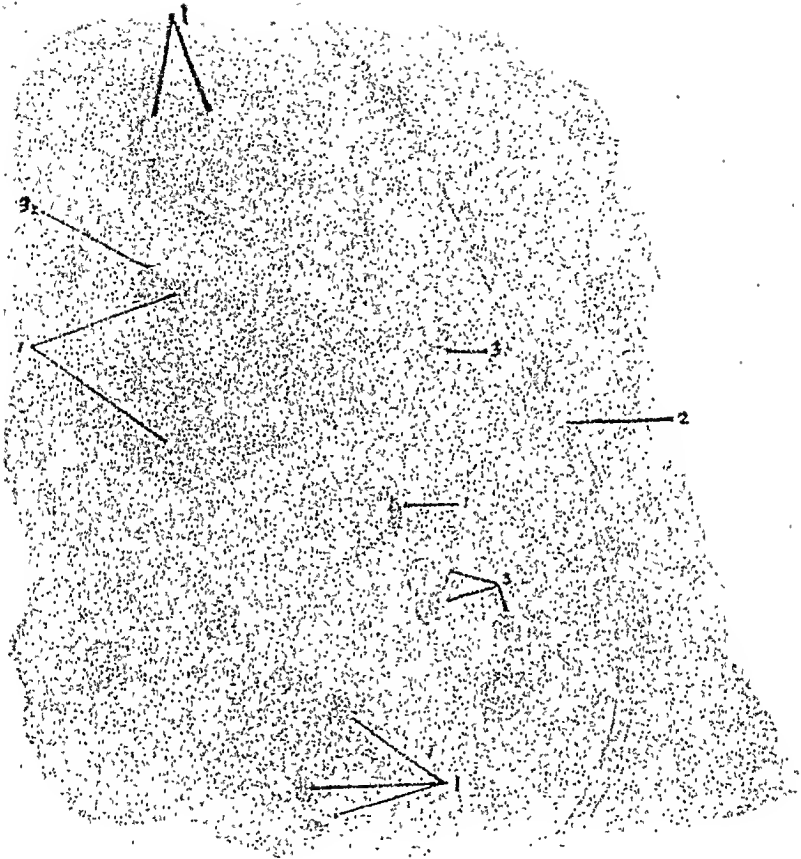
NERVE CELLS CONTAINING NEGRI BODIES.

Hippocampus impression preparation. dog. Van Gieson stain. × 1000. 1, Negri bodies; 2, capillary; 3, free red blood corpuscles. (From Todd and Sanford *Clinical Diagnosis by Laboratory Methods,* W. B. Saunders Co.)

a clean slide into 0.4% salt solution and shake off excess. Press the brain tissue on the cork gently against the prepared slide. A thin section of the brain tissue adheres to the slide and a very satisfactory preparation is thus made.

14. If direct smears and sections are negative, rabbit inoculation should be done. If the brain is fresh and uncontaminated, it may be inoculated at once. Otherwise place in pure glycerol and hold in a refrigerator for 2 or 3 days before inoculation.

Emulsify a small piece of hippocampus major in a small amount of sterile saline and inject about 0.1 to 0.2 c.c. subdurally in an anesthetized rabbit by forcing the needle through the thin bone lying in the depression just posterior to the eye or the intralingual route of injection may be used.

As a general rule the average street virus will cause death in about 16 days, but sometimes requires 3 weeks or longer (paralysis). It is, however, necessary to keep the animals under observation for 60 to 90 days before reporting negative results.

If death occurs within 6 days, it is probably due to bacterial infection and the test should be repeated with glycerolated tissue.

If the animal dies of rabies Negri bodies may be demonstrated in the brain tissue but they are usually smaller and more easily distorted than those produced by street virus.

METHODS FOR THE LABORATORY DIAGNOSIS OF GRANULOMA INGUINALE

1. Granuloma inguinale, granuloma venereum, or groin ulceration is characterized by a swelling which may involve the external genitals, inner surface of the thighs, perineum and anus with involvement of the inguinal lymphatic glands. It occurs more frequently in women.

2. The disease should not be mistaken for lymphogranuloma inguinale, a disease caused by a filtrable virus, for which the Frei intradermal test is employed for diagnostic purposes (See Chapter XXXI).

3. The etiology of granuloma inguinale is uncertain but is probably caused by *Klebsiella granulomatis* (*B. granuloma*), a Friedländer-like organism described by Walker (Fig. 273).

4. The organism occurs in endothelial cells as small oval bodies resembling the "Donovan bodies" of kala-azar.

5. Carefully cleanse the surface of the ulcer with saline solution to remove the pus and then scrape the surface with the edge of a scalpel to obtain tissue cells. Blood and pus should be avoided as much as possible. As the procedure may be painful, it may be necessary to anesthetize the surface with a few drops of novocain or cocain solution. The scrapings should be deep enough to include many tissue cells as superficial scrapings are usually unsatisfactory.

6. Stain the smears with Wright's or Giemsa stains and examine the endothelial cells for the intracellular bodies.

7. If cultures are prepared use Sabouraud's medium on which it occurs as a

short plump coco-bacillus, capsulated, non-motile, non-branching, non-sporulating and gram-negative. Does not liquefy gelatin or produce indole. The colonies are white, moist, convex and viscid.

8. The organism is fatal for guinea-pigs inoculated intraperitoneally with 1 c.c. of culture. Subcutaneous or intracutaneous inoculation of mice and rabbits produce local lesions.
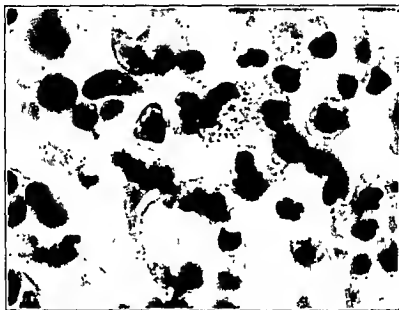
Fig. 273.—KLEBSIELLA GRANULOMATIS OF GRANULOMA INGUINALE

LABORATORY DIAGNOSIS OF KALA-AZAR

1. Examine the blood for anemia due to reduction of erythrocytes and hemoglobin (color index about normal); leukopenia with reduction of polymorphonuclears and eosinophils with increase of lymphocytes and monocytes.

2. Stain smears of blood and examine the polymorphonuclears and monocytes for *Leishmania donovani*; the parasites may rarely occur in erythrocytes. Thick films may be prepared, dehemoglobinized, and stained according to the methods described in Chapter IV for the detection of malarial parasites. Wrights' or the Giemsa stain may be employed.

3. Blood cultures are usually positive. From 0.25 to 0.5 c.c. of blood removed aseptically from a vein at the elbow is placed in 20 c.c. of sterile 1.5% sodium citrate in physiological saline solution. The mixture is gently shaken and allowed to stand overnight in a refrigerator or centrifuged. The supernatant fluid should be decanted and the corpuscles transferred with a sterile pipet to the water of condensation in tubes of N.N.N. medium (1.5% agar with 0.25% glucose and containing about one-third volume of sterile rabbit blood added to the medium at

52° C.). Incubate at 22° to 24° C. (room temperature). Smears are stained with the Wright or Giemsa stains for flagellates.

4. Cultures and smears of the spleen may be made by aspiration with a sterile syringe and are valuable diagnostic aids. Aspiration of the liver is stated to be a safer procedure but frequently yields negative results. Smears of material obtained from bone puncture, excised lymphatic glands, scrapings from bases of artificially produced blisters or ulcers, are stated by Brahmachari to be unsatisfactory.

5. Leishman-Donovan bodies as seen in cells are circular to elliptical in shape, from 2 to 4 micra in diameter, and contain two nuclei, a large oval one at one part of the periphery and a small circular or rod-shaped one (blepharoplast) near or at the opposite part of the periphery (See Fig. 96). This smaller body stains more deeply than the larger one, while the cytoplasm of the parasite stains very dimly, sometimes showing only a faint peripheral rim. Flagellates are seen in cultures (See Fig. 95).

CHAPTER XX

DIAGNOSTIC MYCOLOGICAL METHODS

By EDWIN S. GAULT

In this brief chapter on the laboratory diagnosis of fungus infections, an attempt has been made to give the worker in this field a concise, general technical procedure which may be applied to any material received for mycologic examination. There is also included more specific procedures of diagnosis when the group of suspected fungi is aided by a knowledge of the clinical lesion produced (ringworm, blastomycosis, sporotrichosis, etc.). In addition to diagnostic methods, aids to species identification are included under each group.

GENERAL TECHNIC FOR THE LABORATORY DIAGNOSIS OF THE MORE IMPORTANT FUNGOUS INFECTIONS OF MAN

The laboratory diagnosis of a fungous disease, in general, is accomplished by:

1. Direct examination of material obtained from the lesions, unstained or stained

2. Culture

3. Animal inoculation

4. Special methods

Procedure.—General Instructions.—1. Collection of a suitable specimen.

If the lesions are upon the skin, clean up the affected surface with 70 or 80% alcohol; secure with a sterile instrument diseased hairs, scales or scrapings from the outermost portion of the lesion where the process is active, and place them in a sterile container, such as a Petri dish.

If the lesions are moist, specimens may be obtained from the more active portion of the lesion by scrapings from the surface or by collecting the exudate.

In the case of pustules or abscess-like lesions it is better to aspirate the exudate from unopened lesions with a sterile syringe.

Sputum and feces specimens should be collected as for bacteriological examinations and sent to the laboratory in sterile containers.

In mycosis of the lung, exudate, sputum or tissue fragments may be obtained by bronchoscopy, thus avoiding contamination with organisms of the mouth.

Biopsies for mycologic examination should be taken aseptically and sent to the laboratory in sterile containers. Part of the specimen should be fixed and sectioned for histopathology and the remainder used for mycologic and bacteriologic studies.

2. Specimens for examination should be sent to the laboratory and examined

without delay. Material more than three to four hours old is less apt to give a satisfactory result.

3. Whenever practicable specimens for mycologic examination should be taken by or at least in the presence of the mycologist who will later make or supervise its examination.

4. With every specimen, in addition to the usual data, the following information should be furnished: (a) the nature and source of the material, (b) the time of collection, and (c) the type of fungus suspected by the clinician.

5. Material for mycological examination should be divided into three parts: (a) for direct examination, (b) for culture, (c) for possible animal inoculation and special tests.

Direct Examination.—1. If the specimen is in the nature of hairs, cutaneous scrapings or scales, prepare moist preparations with 20% sodium hydroxide (detailed instructions under ringworm, page 517) and observe the morphology of the mold and its relation to the hair, etc.

From the direct examination of such material in accordance with Sabouraud's "Clinical Classification," the following genera may at once be determined:

Achorion—forms in which the fungus invades the hair but clinically forms a scutulum (a sebaceous, waxy, yellowish secretion) at the junction of the hair and scalp.

Microsporon—forms in which masses of uniform, round cells (spores) are found in the sheath about the hair just beneath the cuticle.

Trichophyton—Sabouraud places under this genus all other forms which invade the hair.

Epidermophyton—This genus is made up of forms which invade the epidermis, but not the hair.

If it is possible to thus determine the genus proceed to identify the species and establish its pathogenicity by culture and animal inoculation, etc.

2. If the specimen is in the nature of pus, exudate, sputum, feces, etc., prepare moist preparations or stained smears and examine for yeast-like fungi or mycelial filaments.

If upon gross inspection of the material or exudate, tiny granules, grayish, sulphur-yellow or black are seen, carefully examine for actinomycetes.

Culture (Modified after Fisher and Arnold).—1. Inoculate several plates of Sabouraud's 4% maltose agar pH 5.2 with several dilutions of the material.

2. Inoculate several tubes of 4% maltose broth pH 5.2 with similar dilutions.

3. Incubate 48 hours or longer at 37.5° C.

4. Make stained smears of suspected colonies and examine under the microscope for yeast-like bodies.

5. Examine the bouillon cultures by stained smears.

6. Obtain pure cultures by inoculating Sabouraud's maltose medium with colonies showing yeast-like forms.

7. Subculture on cornmeal agar plates making deep streaks and allow to grow at room temperature for 48 hours or longer.

8. Subculture on plates or tubes of honey agar and grow at room temperature or in the incubator for 48 hours or longer.

At this stage of examination of fungi having a yeast-like form in primary cultures on Sabouraud's maltose agar or broth, it is possible to distinguish the following genera based upon the presence or absence of mycelial filaments (on cornmeal agar) and the presence or absence of osci (on old cultures):

Key to Genera[1]

YEAST-LIKE FUNGI

(Single-celled organisms reproducing by budding)

(Maltose agar, or broth)
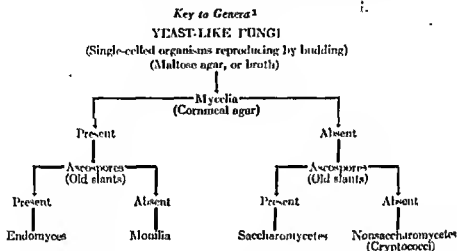
9. Demonstration of ascospores: from old cultures (at least 10 days old) of the fungus on maltose and cornmeal agar prepare smears from the dried top of the fungus and stain with Beauverie stain as follows:

(a) Fix with heat.

(b) Cover with carbol-fuchsin.

(c) Steam gently for 2 minutes.

(d) Decolorize with 25% glacial acetic acid.

(e) Counterstain with methylene blue one-half minute (ascospores are red, vegetative cells blue).

10. To biologically prove the species of the fungus additional cultures are necessary:

(a) Inoculate pure cultures of the fungus on various sugar media with pH adjusted to 5.2—dextrose, maltose, saccharose, lactose, levulose, mannose, galactose, glycerol, trehalose, dextrin and inulin.

(b) Milk (Stovall method of preparation for best results).

(c) Gelatin—liquefaction or type of growth if no liquefaction occurs.

(d) Special media.

A brief consideration of the biologic characteristics of the more important species of fungi will be found in other sections of this chapter under Aids to Species Identification.

[1] After Castellani's biological classification.

Animal Inoculation.—The pathogenicity of the organism may be determined by animal inoculation. Rabbits, guinea-pigs, rats may be used. Inoculations may be made through intravenous, intraperitoneal, cutaneous and subcutaneous routes. For details see additional instructions under individual species.

Special Methods.—Various serologic methods including agglutination, cross-agglutination, agglutinin absorption and complement fixation and in addition other special methods may be conducted.

Glossary of Common Mycologic Terms [2]

Thallus: The actively growing, vegetative organism as distinguished from the reproductive portions.

Hypha: The single thread-like portion.

Mycelium: A group or matted mass of branching hyphæ.

Septa: Divisions of a hypha formed by transverse partitions.

Spores: Cells developed for the propagation of the species.

Conidia: Spores formed directly from the vegetative portion by abstriction, budding or septate division.

Conidiophore: The hypha bearing a spore or group of spores.

Ascospores: Group of spores, usually 4 or 8, enclosed in a sac, or ascus.

Oospores and Zygospores: The spore resulting from the union of two similar spores is a zygospore; if the spores uniting are male (antheridium) and female (oosporangium) the resultant spore is an oospore.

Endospore: A spore formed within an outer envelope.

Blastospore: A spore formed by budding.

Arthrospore: A spore formed of segments of a hypha and released by disarticulation.

Oïdia: Arthrospores of cylindrical form.

Chlamydospore: A large spore, either intercalary or terminal, with tough and frequently double contoured (thick) wall, undergoing encystment.

Thallospore: Any spore formed from the main hypha (or thallus) directly, as in the preceding three.

Sterigma: A short stalk bearing chains of conidia (as in Aspergillus).

Vesicle: The swollen end of a hypha bearing groups of spores.

Columella: The distal end of a hypha forming the supporting center of a sporangium.

Coremium: Bunched groups of conidiophores seen in some species. (Resemble bunch of asparagus.)

Sporangium: A sac containing an indefinite number of spores, usually many, at the end of a hypha.

Sporangiophore: A hypha bearing a sporangium.

Stolon: Runner-like branches of certain fungi (*Rhizopus*).

[2] After Simmons, *Laboratory Methods of the United States Army*, Lea & Febiger, Philadelphia.

Rhizoids: Root-like groups occurring along stolons.

Fuseaux: Fusiform septate spores, produced by certain fungi (Trichophyton).
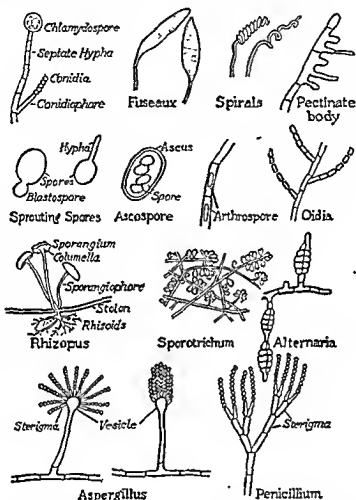
FIG. 274.—TERMINOLOGY USED IN THE DESCRIPTION OF FUNGI

(From Simmons *Laboratory Methods of the United States Army*, Lea and Febiger, Philadelphia.)

Spirals: Terminal coils seen in some species.

Pectinate bodies: Comb-like structures formed by some fungi.

METHODS FOR THE IDENTIFICATION OF THE RINGWORM FUNGI

Collection of a Suitable Specimen.—1. Clean up the affected surface with 70 or 80% alcohol; secure with a sterile instrument diseased hairs, scales or scrapings from the outermost portion of the lesion where the process is active, and place them in a sterile container, such as a Petri dish.

2. If the lesions are moist, specimens may be obtained from the more active portions by scrapings from the surface or by collecting the exudate.

3. Material for examination should be sent to the laboratory and examined without delay. Specimens over 3 or 4 hours old are less apt to give satisfactory results.

4. If possible, divide the specimen in three parts: (*a*) for direct examination, (*b*) for culture, (*c*) for possible animal inoculation.

Direct Examination.—1. Prepare a slide by outlining with vaseline a circle or square slightly smaller than the coverglass.

2. Place some hairs, scrapings, scales, etc., within; cover with several drops of 20% sodium hydroxide. Carefully cover with a coverglass.

3. Allow the hydroxide to act for 20 minutes to a half hour or until the tissue elements have been dissolved, thus leaving the fungi free for observation.

4. Examine the hair or other material by reduced transmitted light under low and high power for the presence of fungi.

5. Observe the presence of molds within the hair or outside the hair, or both within and without.

Culture.—1. If the material to be examined consists of dried scrapings, soak for an hour in alcohol to destroy as many contaminating bacteria as possible.

Caution.—The alcohol used for this purpose should be "controlled" and known to be free from spores or molds.

2. Drain off the alcohol.

3. Inoculate with a sterile inoculating needle 4 tubes of Sabouraud's medium.

4. Inoculate each slant at three points, slightly breaking the surface of the medium to introduce the spores.

5. Place some tubes in the incubator (37.5° C.) and allow others to grow at room temperature.

6. Observe the cultures daily, but do not open the tubes unless definite growth is seen.

7. When growth occurs observe characteristics of the colony.

8. Subculture on Sabouraud's maltose agar medium, and transfer a portion of the colony to a slide.

9. Add a few drops of salt solution, or preferably, standard mounting fluid:

2% solution potassium acetate	300 c.c.
Glycerin	120 c.c.
95% alcohol	180 c.c.

10. Cover with a coverglass and examine with high and low power.

Animal Inoculation.—Animal inoculation is not usually resorted to in the diagnosis of ringworms. However, a portion of the material may be inoculated by scarification or subcutaneous injection. The mouse, guinea-pig and rabbit are the animals most frequently used.

AIDS TO THE IDENTIFICATION OF THE RINGWORM FUNGI

The following outline will be of assistance in determining the genus of the fungus, according to Sabouraud's classification of ringworm fungi and closely allied species:

Achorion.—Sabouraud places forms in which fungus invades the hair but clinically forms a scutulum (a sebaceous, waxy, yellowish secretion) at the junction of the hair and scalp under the genus Achorion.

Microsporon.—The microsporon includes those forms in which masses of uniform, round cells (spores) are found in the sheath about the hair just beneath the cuticle.

Trichophyton.—Sabouraud places under this genus all other forms which invade the hair.

Epidermophyton.—The genus epidermophyton is made up of forms which invade the epidermis but not the hair.

There are a number of species of ringworm pathogenic to man, producing a variety of cutaneous lesions. The most important species are summarized as follows, following Sabouraud's clinical classification (modified from Gay):
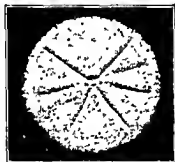
Fig. 275.—Microsporon Lanosum

Six weeks' culture on Sabouraud's test medium; one-half natural size (Hopkins).

Fig. 276.—Fuseaux of Microsporon Lanosum ×200

1. Microsporon Lanosum.—This is one of the frequent causes of ringworm of the scalp and skin acquired from animals.

In the microscopic examination of the diseased hair small, round spores form a sheath about the hair just beneath the cuticle. On Sabouraud's maltose or honey agar large, flat, white (later tan) colonies are produced (Fig. 275). Material taken from the surface of the culture contains numerous lenticular fuseaux, which are thick-walled and provided with spines. See Fig. 277. Microconidia are also produced along the sides of the mycelium. Chlamydospores occur and are found with thick walls (Fig. 276).
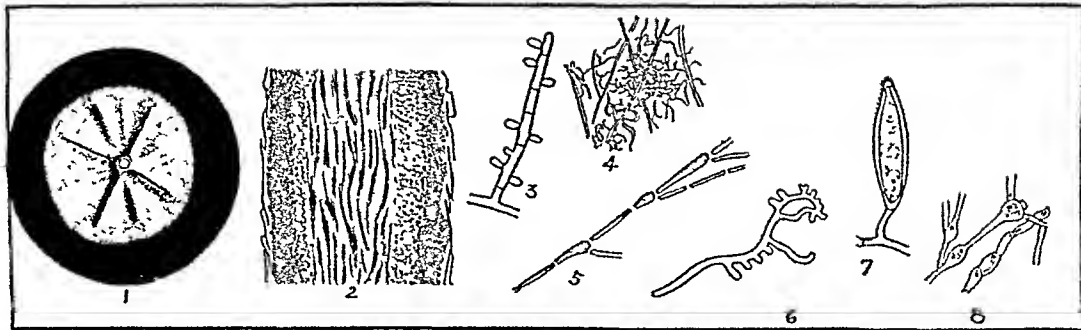
MICROSPORUM AUDOUINI

*1 White colony, velvet surface   2 Infected hair   3 Microconidia   4 Mycelial tangle
5 Racquet mycelium   6 Pectinate body   7 Rudimentary fuseau   8 Chlamydospores*
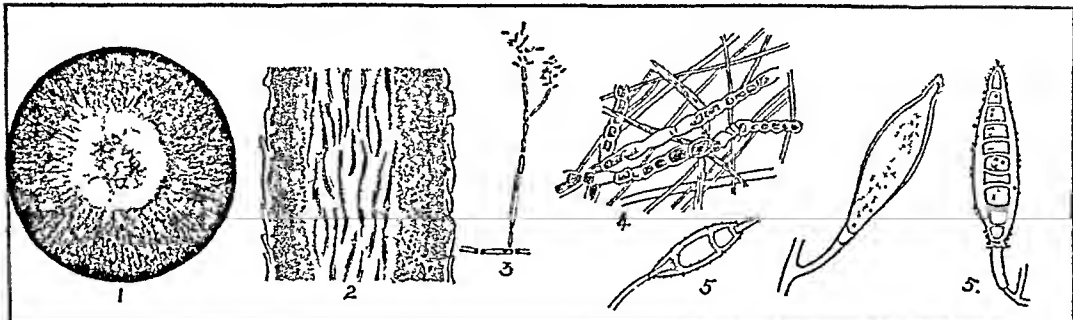
MICROSPORUM LANOSUM

1 Tan colony, wooly surface   2 Infected hair   3 Microconidia   4 Chlamyodspores   5 Fuseaux

TRICHOPHYTON GYPSEUM

*1 White colony, powdery surface   2 Infected hair, (Ectothrix)   3 Conidial cluster   4 Spirals   5 Fuseaux
6 Microconidia   7 Nodular organ*
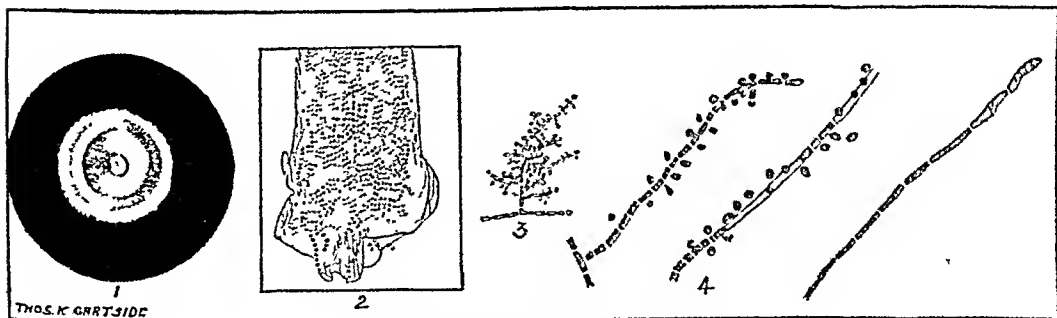
TRICHOPHYTON CRATERIFORME

1 White colony, velvet surface   2 Infected hair (Endothrix)   3 Conidial cluster   4 Microconidia

FIG. 277.—RINGWORM FUNGI. (From Sabouraud's *Les Teignes.* Courtesy of Masson and Cie.)

2. **Microsporon Audouini.**—This organism is one of the most common causes of ringworm of the scalp in the United States. The infected hair is similar in appearance to that of *M. lanosum*.

On Sabouraud's lactose or honey agar, velvety white or buff colonies are produced which are slow in growth. A knob usually forms in the center with radial grooves toward the margin.

Microscopically, cultures show only a few rudimentary fuseaux but are characterized by peculiar structures known as "pectinate bodies." These are small parallel protuberances extending out from one side of swollen hyphal branches resembling a comb. Other features are the presence of chlamydospores, racket mycelia and microconidia. (See Fig. 277.)

(a) **Endothrix Trichophytons.**—These are important causes of ringworm in the hair. The spores are large, arranged more or less in rows, entirely within the

FIG. 278.—HAIR RIDDLED WITH RINGWORM FUNGUS, MEGALOSPORON VARIETY
(From Park, Williams and Krumwiede, *Pathogenic Microörganisms*, Lea and Febiger.)

hair. They are differentiated by their cultural characteristics on Sabouraud's lactose or honey agar. Several species are found.

1. *Trichophyton crateriforme (T. tonsurans).*—This organism forms a white, creamy colony with powdery surface, the central portion showing a crater.

Microscopic examination of the culture shows rather characteristic conidial clusters. Chlamydospores are abundant. Microconidia are present. (See Fig. 277.)

2. *Tricophyton acuminatum (T. sabouraudii).*—This fungus is quite similar to that of T. crateriforme.

3. *Trichophyton violaceum.*—This is frequently responsible for ringworm in Italy and Russia. On Sabouraud's medium deep violet colored colonies are produced having a smooth, waxy surface, sometimes with grooves from the center to the periphery.

Microscopic examination of portions of the culture reveals a mycelium with many short, crooked segments, and frequent chlamydospores. See Fig. 279.

(b) **Ectothrix Trichophytons.**—The infected hair shows the spores arranged in chains composed of uniform sized spores between the sheath and cuticle of the hair.
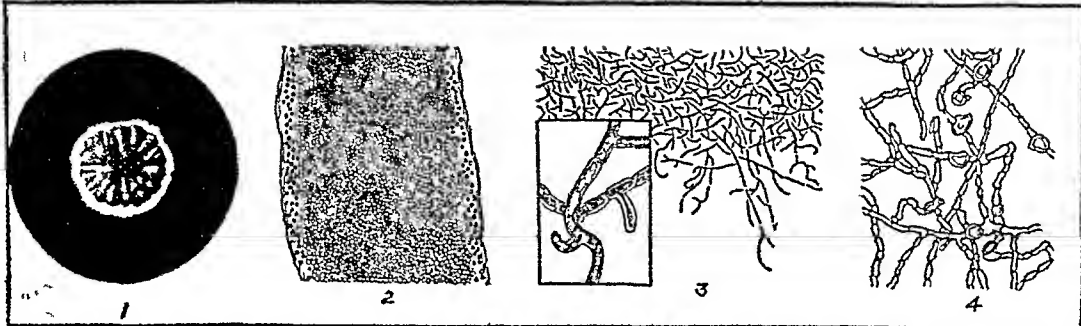
TRICHOPHYTON VIOLACEUM

1 VIOLET GLABROUS COLONY   2 INFECTED HAIR (ENDOTHRIX)   3 YOUNG MYCELIUM   4 OLD MYCELIUM WITH VESICULAR CELLS
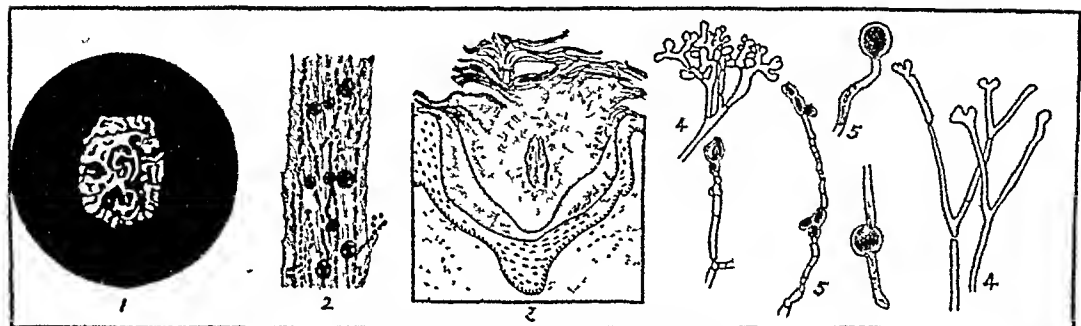
ACHORION SCHÖNLEINII

1 TAN GLABROUS COLONY   2 INFECTED HAIR   3 SCUTULUM: (A) HAIR (B) FUNGUS (C) EPIDERMIS   4 CHANDELIERS   5 CHLAMYDOSPORES
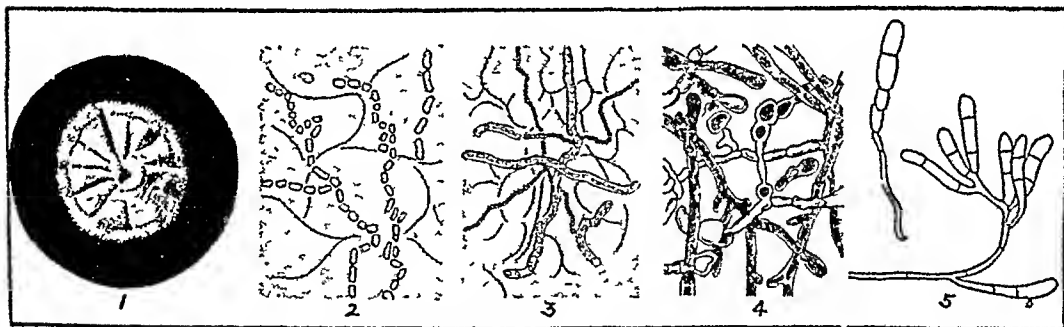
EPIDERMOPHYTON INGUINALE

1 GRAY GREEN DOWNY COLONY   2-3 MYCELIUM IN EPIDERMAL SCALE   4 CHLAMYDOSPORES   5 FUSEAUX
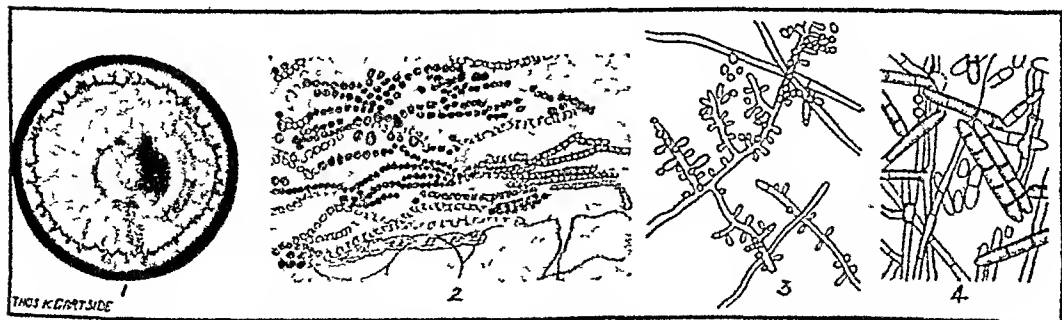
EPIDERMOPHYTON (TRICHOPHYTON) RUBRUM

1 WHITE DOWNY COLONY WITH GLABROUS PURPLE BORDER   2 FUNGUS IN NAIL   3 MICROCONIDIA   4 FUSEAUX

FIG. 279.—CHARACTERISTIC TYPES OF RINGWORM FUNGI

Trichophyton violaceum, Achorion schonleinii, Epidermophyton inguinale, Epidermophyton

1. *Trichophyton asteroides* (**Gypseum, T. mentagrophytes**).—Upon Sabouraud's medium a creamy white growth occurs. The growth is rapid, the surface is powdery, and the edge of the colony is uneven and ray-like (Fig. 277).

Microscopic examination reveals the presence of atypic or rudimentary fuseaux, large clusters of conidia, numerous spirals, microconidia and nodular organs.

2. *Trichophyton rosaceaeum*.—The culture grows slowly on Sabouraud's medium, producing a white, folded disk-like colony which becomes pink or reddish with age.

Microscopic examination reveals rudimentary fuseaux and long thyrses.

(c) **Neoendothrix Trichophytons.**—Trichophyton cerebriforme and Trichophyton plicatile.—These are very rarely found, and resemble in culture the *Trichophyton crateriforme*.

1. **Epidermophyton inguinale (T. cruris).**—This fungus is commonly the cause of ringworm of the feet and eczema marginatum of the groin.

Examination of the infected skin reveals that the fungus has long interlacing filaments made up of oblong double contour cells.

On Sabouraud's medium the colonies develop slowly. Due to rapid pleomorphism, they may be grayish white, lemon yellow or greenish.

Microscopic examination reveals characteristic smooth-walled fuseaux (Fig. 280) which are found in clusters. Conidia are not found (Gay). Fig. 279.

2. **Epidermophyton rubrum.**—This is another closely allied species. See Fig. 279.

3. **Epidermophyton (Ctenomyces) interdigitale (Gay).**—This is probably identical with the white fungus of Kaufmann-Wolf and similar to the T. niveum group. The culture is at first pure white, later gold. Some strains

Fig. 280.—Fuseaux of Epidermophyton Inguinale ×200.

show a reddish color. It is made up of tangled hyphae, mostly sterile but with few scattered aleurospores. Macroconidia may be seen in old cultures.

METHOD FOR THE LABORATORY DIAGNOSIS OF FAVUS

In this condition, the diseased hairs are surrounded by yellowish, waxy crusts at the base of the hair called "scutula."

The diagnosis is made by examining a number of broken off diseased hairs under the microscope and by culture for the fungus.

For collection of specimen, technic of preparation and culture, see Procedure for the Identification of Ringworm, page 516.

The most common organism responsible for favus is *Achorion schoenleinii*.

Achorion schoenleinii.—Microscopic examination of the diseased hairs will reveal large, irregular-shaped spores occurring within the hair and irregularly arranged. Long hyphae extend out beyond the hair into the sealp. These hyphae may be simple or segmented.

Culture on Sabouraud's medium at the end of one or two weeks shows yellowish, shiny colonies, later becoming wrinkled irregularly and darker in color. The organism rapidly becomes pleomorphic.

Microscopic examination of portions of the fungus shows numerous club-shaped terminal branches, many of which are notched at the tip. A characteristic formation is the appearance of clusters of such clubs. See Fig. 279.

METHODS FOR THE LABORATORY DIAGNOSIS OF PITYRIASIS VERSICOLOR (TINEA VERSICOLOR, CHROMOPHYTOSIS)

This skin disease is produced by the *Microsporon furfur*.

The laboratory diagnosis is accomplished by: (1) direct examination of scrapings from the lesions for the organism, stained and unstained; (2) culture.

The direct examination and culture are the same as under ringworm, page 516.

METHODS FOR THE LABORATORY DIAGNOSIS OF BLASTOMYCOSIS

There are two forms of blastomycosis: (1) Blastomycotic dermatitis and (2) the general or systemic variety.

1. Secure a suitable specimen.

In the cutaneous variety, pus should be collected from the miliary abscesses present at the edge of the lesion, and placed in a sterile container. In the systemic variety, specimens of tissue or pus from the diseased area will be suitable. When this is impossible, a specimen from metastatic cutaneous lesions will suffice.

2. Place a small amount of the pus or necrotic tissue on a slide, and add a few drops of 10 or 20% sodium hydroxide. Cover with a coverglass.

3. When cleared, examine for the yeast-like fungi, the blastomyces hominis (Fig. 281).

This organism is oval or rounded in shape, measures from 10 to 20 micra, and reproduces by budding. The presence of the parent cell with bud gives rise to the so-called "figure eight" arrangement of the fungus. Each organism is surrounded by a hyalina capsule which is highly refractile. Within, the cytoplasm is granular. The organism may be stained with methylene blue or Wright's stain.

Culture.—1. Inoculate some of the pus upon Sabouraud's maltose or honey agar and corn meal agar.

The organism grows readily on the various laboratory media. It is easily overlooked or lost in culture due to numerous bacteria usually present in the pus, which tend to outgrow the fungus.

2. Inoculate at least two tubes, and incubate at 37° C. and 22° C.

3. After from 2 days to 2 weeks, examine carefully for small white colonies resembling *Staphylococcus albus*.

4. Examine fresh and stained (methylene blue) preparations for one of the blastomyces.

Cultural Characteristics of Blastomyces hominis.—This organism or group of organisms grows readily on culture media, producing on Sabouraud's or corn

meal agar a small, white, glistening, rounded colony, which after a week or ten days becomes wrinkled and slightly brownish in color. Gelatin is not liquefied. On bouillon the medium remains clear, and a stringy sediment is found at the bottom. Most strains of the group do not ferment carbohydrates.

Microscopic examination of a portion of colony reveals the same morphology as described above with occasional "figure eight" formation, and in some cultures early, and in others late, the formation of irregularly branched septate hyphae. These filaments are most numerous when the cultures are grown at room temperature.

Examination of Tissue.—Tissue obtained by biopsy or autopsy should be run through in the regular way (see Chapter

Fig. 281—BLASTOMYCES SHOWING BUD-DING FORMS IN PUS FROM A LESION IN MAN

(From Gilchrist)

XXXVIII), and stained with hematoxylin and eosin, polychrome methylene blue, or theonin. When stained with hematoxylin and eosin the capsule usually remains unstained, the protoplasm is usually basic and shows deep blue-staining granules.

METHODS FOR THE LABORATORY DIAGNOSIS OF COCCIDIOIDAL GRANULOMA

This disease produces lesions primarily involving the skin, which may later spread to lymph nodes, bones, etc. Lesions are nodular and may break down forming ulcers and abscesses. Lesions may be pulmonary, and the organism may be found in the sputum.

The *laboratory diagnosis is made by* (1) direct examination of the exudate from the lesions for the fungus, (2) culture, (3) tissue biopsy, (4) animal inoculation.

Direct Method.—1. Secure a suitable specimen.

Pus from one of the cutaneous lesions or a tissue biopsy are suitable, and should be taken, exercising aseptic precautions, and sent to the laboratory in a sterile test tube or Petri dish.

If sputum, the patient should rinse the mouth well, preferably with 50% alcohol before the specimen is expectorated.

2. Place a small amount of the pus or necrotic tissue on a slide and add a few drops of 10 to 20% sodium hydroxide. Cover with a coverglass.

3. When cleared, examine for the fungus, *Coccidioides immitis*.

This organism, if present, will be seen as small round bodies, measuring 5 to 50 micra in diameter, each surrounded by a refractile capsule. Cytoplasm is granular. No buds are seen, which is the chief point of differentiation from the blastomyces (Fig. 282).

Cultural Method.—1. Inoculate some of the pus upon Sabouraud's maltose or honey agar and corn meal agar. The organism also will grow on the ordinary laboratory media.

2. Inoculate at least 2 tubes, and incubate one at 37° C. and the other at 22° C.

3. Examine carefully for the white glossy colony, lightly covered with a dusty white layer of hyphae, which should appear in from 2 to 7 days. The color tends to become darker as the organism grows older. On Sabouraud's honey agar the color of old colonies is greenish black. Gelatin shows slow liquefaction, litmus milk gradually peptonizes, and the sugars are not fermented.

Microscopic examination of portions of the culture show many round forms from 2 to 8 micra in diameter, from which

Fig. 282.—Coccidioides, Round and Sporulating Forms in a Giant Cell in a Lesion in Man

(From Rixford and Gilchrist)

spring septate and branched hyphae. Chlamydospores and conidia are said to occur in all cultures.

Animal Inoculation.—The organism is pathogenic to animals (rabbits and guinea-pigs).

Inoculate a small quantity of the pus subcutaneously or intraperitoneally into a guinea-pig and examine the lesions which develop for the organism.

METHODS FOR THE LABORATORY DIAGNOSIS OF SPOROTRICHOSIS

In this disease, the infection is chronic in nature, and the lesions are found mostly in the skin or subcutaneous tissues.

The laboratory diagnosis is established by (1) direct examination of the exudate from cutaneous lesions, (2) cultural methods, (3) by animal inoculation.

Direct Method.—1. Technic of examination is the same as for coccidiodal granuloma (see page 524).

2. Direct examination of the pus for the demonstration of *Sporotrichum schencki* reveals elongated oval cells, ranging from 3 to 10 micra in length and from 1 to 3 in width. These cigar-shaped spores in the tissues may be found within giant cells (Fig. 283).

Fig. 283.—Sporotrichum Schenki

Spores in lesion in testis of rat. (From Hopkins)

Fig. 284.—Sporotrichum Schenki, Spores in Lesion in Testis of Rat

(From Hopkins)

The direct examination of the pus, however, is usually unsuccessful, even if the pus is cleared with 10 to 20% sodium hydroxide. The diagnosis is usually made by culture.

Culture.—The culture method is the same as for Coccidioides.

In 4 or 5 days on Sabouraud's maltose and honey agar the *Sporotrichum schencki* grows in the form of tiny fringed grayish colonies. As the colony grows larger, the center becomes wrinkled, and is surrounded by a border of rather regular radial grooves. The color darkens with age.

Microscopic examination of portions of the culture shows many fine branching hyphae about 2 micra in diameter, the branches terminating in groups of oval or pear-shaped spore-like projections, the arrangement being somewhat like a clover leaf. See Fig. 284.

Animal Inoculation.—Some of the exudate may be injected subcutaneously or intraperitoneally into a male rat. The organisms tend to form localized lesions in the testes and joints. Cutaneous papules may be produced along the tail.

METHODS FOR THE LABORATORY DIAGNOSIS OF MONILIASIS
(INCLUDING THRUSH AND SPRUE)

The monilia may produce lesions in the mouth (thrush), in the intestine (tropical sprue), in the vaginal mucous membrane, in the bronchi and lung, in the meninges, and in the skin (erosio interdigitalis).

The laboratory diagnosis is established by (1) direct examination of scrapings or exudate from the lesions for the fungus, (2) culture, (3) animal inoculation.

Direct Examination.—1. *Obtain a suitable specimen.*

From the mouth (in thrush) or the vagina, secure scrapings of a portion of the white patches on the mucous membrane. In cutaneous moniliasis, secure scrapings or exudate from the diseased skin. In intestinal moniliasis (sprue), a portion of the feces should be examined. In meningeal moniliasis, the spinal fluid is to be examined. In pulmonary moniliasis, the organisms may be found in the sputum.

2. Place a small amount of pus or necrotic tissue on a slide and add a few drops of 10 to 20% sodium hydroxide. Cover with a coverglass.

3. When cleared, examine for *Monilia albicans* (*Oidium albicans*). In fresh preparations the organism is composed of oval, yeast-like cells and mycelial filaments. The yeast-like cells are also found laterally along the branches and may lie free (see Figs. 285 and 286).

Caution: A number of nonpathogenic monilia are found upon the skin and in the intestines, and must be differentiated; *Monilia candida, Monilia parapsilosis,* and *Monilia krusei* are among the most common. (See Fig. 287.)

Culture.—1. Inoculate at least 4 tubes with some of the material, slightly breaking the surface, upon Sabouraud's honey agar and cornmeal agar, and place in incubator at 37° C. and 22° C.

Note: In sprue, a small amount of feces should first be considerably diluted with bouillon or salt solution, and then the media touched at 4 points with a drop

of diluted feces by means of an inoculating needle. Bacteria will first appear and will be overgrown later by the fungus.

2. *Examine carefully after a few days for creamy, moist colonies with smooth surface.*

After a week or two, the center of the colony shows a honeycombed appearance. The margins are first smooth and later become lobulated. Most strains do not liquefy gelatin. In bouillon the broth is clear with flocculi at the bottom. Dextrose, levulose and maltose are fermented.
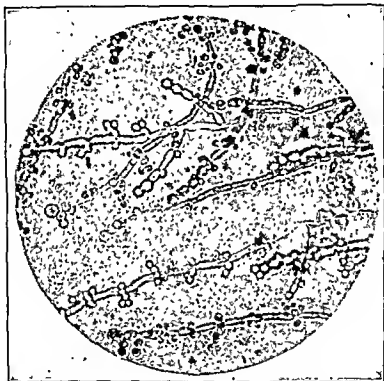
Fig. 285.—Monilia Albicans, Unstained, Showing Mycelium Spores and Yeast-like Cells
(From Benham)

Microscopic examination of a portion of the young colony from honey agar reveals many yeast-like oval cells 5 to 6 micra in diameter, which are highly granular, showing budding at one or both ends. A few rudimentary filaments may also be seen. Older cultures show more filaments.

On cornmeal agar hyphae are developed early, showing clumps of yeast-like budding forms occurring at points of juncture, together with large ball-like clusters (Fig. 287).

Animal Inoculation.—Rabbits may be injected intravenously with 1 c.c. of a 1:1000 suspension of the suspected organism from culture, and if the organism

is parasitic, it will produce death in 4 or 5 days, with miliary abscesses scattered over the viscera, the serous membrane, and kidney. (Gay.)

Guinea-pigs may be inoculated by superficial scarification. A mild dermatitis will result in from 1 to 3 weeks. The organism can be identified in the scrapings from the lesions.

METHODS FOR THE LABORATORY DIAGNOSIS OF DISEASES DUE TO ASPERGILLI AND PENICILLIA

Many species of *Aspergillus* and *Penicillia* are found in Nature, and are among the commonest saprophytic molds. Their spores are practically ubiquitous, and therefore they may contaminate culture media, various uncovered specimens, open wounds, etc.

Many species have been described as pathogenic which are probably accidental, secondary contaminants.

One species of *Aspergillus*, the *Aspergillus fumigatus*, is pathogenic, producing pulmonary aspergillosis.

Collection of specimen, examination of culture, and animal inoculation should be conducted as described under Coccidia, the

Fig. 286.—Oidium Albicans
(After Zettnow)

Aspergillus being associated with pulmonary and cutaneous aspergillosis.

These molds are quite complex, are of large size, and are best studied by carefully removing a portion of the medium upon which the mold is growing. This may be placed on a slide and examined with the low power of the microscope.

Aspergillus.—There are many species of *Aspergillus*, showing on culture media various colors, and developing a low velvety growth, black, white, yellow, etc. The *Aspergillus fumigatus* produces a brownish colony on neutral or alkaline media, and slightly greenish colony on acid media.

On microscopic examination, the mycelium is septate. Numerous aerial hyphae terminate in an expanded portion from which arise many short conidiaphors, at the end of which are a chain of 4 or more round conidia. Ascospores are sometimes seen (See Fig. 274).

Penicillium.—The *Penicillium* is much smaller. When fully developed it produces a dirty greenish velvety growth. If a portion is examined under the microscope a septate mycelium will be found with aerial hyphae which branch and rebranch, forming conidiaphors which terminate in 6 to 8 spores (See Fig. 274).

Mucors.—The *Mucors* are a group of nonpathogenic molds which are likewise commonly met with in contaminations of culture media, etc. They are much larger than the *Aspergilli* and *Penicillia*, and on culture media their hyphae resemble cotton-fibers, and the colonies look somewhat like tufts of cotton.

From the main mass of the mold, aerial hyphae extend terminating in a black, tiny spore-case visible with the naked eye.

SACCHAROMYCES CEREVISIAE
Budding cells (A)   No mycelium   Ascospores (B).
Honey Agar   Corn-meal Agar

WILLIA ANOMALA
Budding cells (A)   Rudimentary mycelium (C)
Hat shaped ascospores (B)
Honey Agar   Corn-meal Agar
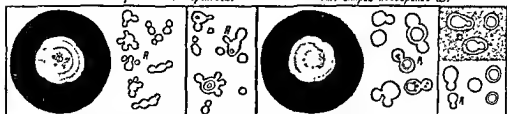
CRYPTOCOCCUS GLABRATUS
Budding cells (A)   No mycelium   No ascospores
Honey Agar   Corn-meal Agar

CRYPTOCOCCUS HOMINIS
Budding cells (A)   No mycelium   No ascospores
Capsule (D)   Gelatinous colony
Honey Agar   Corn-meal Agar

MONILIA ALBICANS
Budding cells (A)   Rudimentary mycelium (C)   Mycelium (E)   No ascospores   Chlamydospore (F)   Clusters (G)
Honey Agar   Corn-meal Agar

MONILIA PARAPSILOSIS
Budding cells (A)   Mycelium (E)   No ascospore
Honey Agar   Corn-meal Agar

MONILIA KRUSEI
Budding cells (A)   Mycelium (E)   No ascospores
Honey Agar   Corn-meal Agar
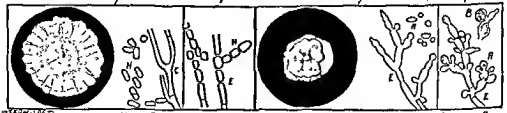
MYCODERMA LACTIS
No buds   Arthrospores (H)   Mycelium (E)   No ascospores
Honey Agar   Corn-meal Agar

ENDOMYCES FIBULIGER
Budding cells (A)   Mycelium (E)   Hat shaped ascospores (B)
Honey Agar   Corn-meal Agar

Under the microscope it will be seen to be made up of a covering or perithecium containing a large number of small refractile spores.

If the mold is examined under a coverglass the trauma will fracture the spore-case, and it will be seen as a collapsed capsule with the spores outside. Zygospores, the result of the fusion of lateral buds of adjacent plants, may be encountered.

METHODS FOR THE LABORATORY DIAGNOSIS OF ACTINOMYCOSIS

Actinomycosis is an infectious, granulomatous disease of animals, and occasionally of man. The lesions may occur about the mouth and jaw, in the skin, the subcutaneous tissue, the lung, and occasionally other viscera.

The *laboratory diagnosis* is accomplished by: (1) direct examination of the material for the actinomyces, unstained and stained, (2) culture.

Direct Examination.—1. Secure a suitable specimen.

If the lesions have broken down, discharges of pus or necrosing tissue may be collected and sent to the laboratory in a sterile container.

It is preferable for culture, to aspirate an unopened, softened lesion, which will be free from contaminating bacteria.

Remove tissue by biopsy and send to laboratory in a sterile container.

In pulmonary actinomycosis, the sputum is examined.

2. Spread the pus or material in a thin layer over the bottom of a sterile Petri dish. Salt solution may be added if necessary.

3. Examine with the naked eye or hand lens for small grayish or sulphur-yellowish granules, less than a millimeter in diameter. These granules may be colonies of actinomyces.

4. By means of a platinum loop, place a granule on a slide, and cover with a coverglass. Press out gently (Fig. 288).

5. If the structural details are obscure, place 2 or 3 drops of 20% sodium or potassium hydroxide on the granule before covering.

If the granules are calcified, add a drop of concentrated acetic acid. This will remove the calcium, and make possible examination.

6. Examine under the low power of the microscope, 16 mm. and 10 x ocular, with the light somewhat diminished.

If the granule is the *Actinomyces bovis* or the *Actinomyces hominis*, the center will appear darker and made up of interlacing mycelia, which end in radially arranged terminals (the rays). These rays are closely packed together, are pear- or club-shaped, and are known as "the clubs."

7. The cover-glass can now be removed, the specimen dried and stained by Gram's method as follows:

FIG. 287.—CHARACTERISTIC TYPES OF YEAST-LIKE FUNGI

Ascomycetes: 1, saccharomyces and Willia. No mycelium; reproduces by buds and ascospores; 2, endomyces. Forms mycelium, reproduces by buds and ascospores.

Fungi imperfecta: 1, cryptococcus. No mycelium. Reproduces by buds. No ascospores. 2, Monilia. Forms mycelium, reproduces by buds. No ascospores.

Colonies on honey agar are ½ natural size except Mycoderma lactis which is ¼ natural size. Drawings ×500 except Mycoderma lactis and endomyces fibuliger which are ×250. (From Gay et al., *Agents of Disease and Host Resistance*, Charles C. Thomas, Springfield.)

Stained Smears.—1. Prepare thin smears of material, particularly from crushed granules just described.

2. Stain by Gram's method.

3. The central mycelium of the colony will stain gram-positive, the clubs or bulbous ends will stain gram-negative, and such a gram-reaction is diagnostic of the *Actinomyces* (Fig. 289).

Cultural Method.—1. Secure a number of granules, wash with salt solution

FIG. 288.—ACTINOMYCES GRANULE CRUSHED BENEATH A COVER GLASS
(After Wright and Brown)
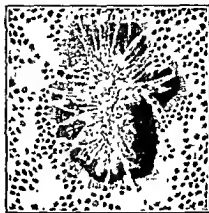
FIG. 289.—ACTINOMYCOTIC GRANULE IN PUS, STAINED TO SHOW THE MYCELIUM
(From Kolle and Hetsch)

and crush. If granules are not found, inoculate a portion of the specimen submitted.

2. Inoculate 8 or 10 tubes of glucose agar (1% glucose glycerin agar pH 7.3 to 7.6 preferred) or blood agar.

FIG. 290.—BRANCHING FILAMENTS OF ACTINOMYCES
(After Wright and Brown)

3. Culture half the tubes anaerobically; the others aerobically in an incubator at 37° C.

4. Examine after 4 or 5 days for colonies of the fungus. At first the colonies are small, dry, opaque, white or grayish in color, adherent, and with a somewhat irregularly roughened surface.

5. Subculture typical colonies to obtain pure culture for use in biologically proving the organism.

6. After subculture, prepare smears and stain by Gram's method.

The organism is Gram-positive, and

appears as a number of dichrotomously-branched filaments. "Clubs" are not found.

If no growth occurs in 2 or 3 weeks, cultures may be considered negative.

7. To biologically prove the organism, inoculate duplicate sets of the pure culture on various laboratory media, and grow both anaerobically and aerobically in an incubator at 37° C.

Cultural Characteristics.—*Actinomyces Bovis.*—*Actinomyces bovis* grows in anaerobic environment, the growth being greatest in stab cultures and shake cultures at a depth of 1 to 2 cm. It produces acid and no gas on lactose, maltose. glucose and salicin. Indol shows negative result. Nitrates are reduced. Litmus milk is not changed, gelatin shows slow liquefaction. Löffler's blood serum is not pitted.

Actinomyces Hominis.—*Actinomyces hominis* is similar to *A. bovis*, but is aerobic. Sugars are not fermented; on litmus milk there is slow peptonization with alkaline reaction. Gelatin and Löffler's blood serum are not liquefied.

Many *Actinomyces* are found growing in nature which are nonpathogenic, and may be encountered.

METHODS FOR THE LABORATORY DIAGNOSIS OF MYCETOMA (MADURA FOOT)

Mycetoma is a suppurating granuloma of the foot, closely related to actinomycosis.

1. The securing of specimen, technic of examination and culture are similar to those described under Actinomycosis.

The granules found in the pus of Madura foot are usually black, although they may be yellowish. greenish or red.

A large number of fungi have been isolated, in addition to 13 species of *Actinomyces,* many of which are saprophytes.

CHAPTER XXI

METHODS OF EXAMINATION OF THE SKIN AND MUCOUS MEMBRANES FOR ANIMAL PARASITES

By EDWIN S. GAULT

CLASSIFICATION

I. **Protozoa.**

Leishmania tropica, producing oriental sore (Delhi boil). Examine stained smears of material from the lesions for the intracellular Leishman-Donovan bodies. See page 510.

Leishmania braziliensis, causing "espundia" in South and Central America. Diagnosis made by examining stained smears of material from the mucocutaneous lesions for the intracellular Leishman-Donovan bodies. See page 510.

II. **Helminthes.**

Onchocerca volvulus (Filaria volvulus), producing a form of filariasis in Africa and South America. Diagnosis is established by demonstration of the microfilaria from the subcutaneous nodules by aspiration. Microfilaria volvulus measures 325 micra. It is an unsheathed embryo. The body substance contains numerous nuclei except at the tail end.

Dracunculus medinensis (Filaria medinensis), commonly known as the guinea-worm. The adult female inhabits the subcutaneous tissue of the lower extremity of man. The infestation is common in Asia, Africa, South America and the West Indies. The diagnosis is usually made clinically or by the appearance of the worm within the subcutaneous tissue. The larvae, however, may be collected after the worm has matured, and examined for identification.

Enterobius vermicularis, seatworm. Examine the skin of the perianal region for ova and parts of the adult worm of the parasite.

III. **Arthropoda.**

In this group many species are met with of medical interest. Most, however, do not result in an infestation of the skin, but are important as vectors or invertebrate carriers of bacterial, protozoal, virus or rickettsial diseases. The paragraphs that follow include only members spending part or all of their life cycle within or upon the skin. Brief mention will be made of the more important species, with methods of diagnosis. For more complete information as to biological characteristics, the reader is referred to textbooks on medical zoology and entomology.

IDENTIFICATION OF MITES

Sarcoptes scabiei, the itch mite
Trombicula irritans, harvest mite
Trombicula akamushi, vector of tsutsugamushi fever
Liponyssus bacota, tropical rat mite
Trombicula holosericeum, the European harvest fly
Pediculoides ventriculosis, the grain mite
Glyphagus domesticus, sugar mite.

Laboratory Diagnosis.—A simple and rapid method for detecting skin mites is carried out by means of a scraping in paraffin oil by the method of Benbrook:

1. Sterilize a scalpel or other scraper in alcohol or gas flame. Cool by dipping into water. Dry.

2. Place a drop of paraffin oil in the center of a microscope slide.

3. Dip the scalpel in the paraffin oil drop (an oily scraper will pick up a specimen more easily than will a dry scraper).

4. Pinch a fold of skin showing lesions, between the thumb and forefinger, and
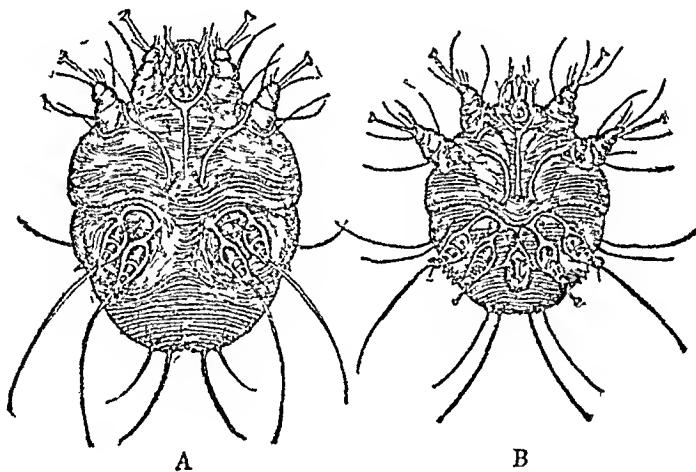
Fig. 291.—Acarus Scabiei (Ventral Surface)

A. Female. B. Male. ×100. (From Schamberg, *Diseases of the Skin and Eruptive Fevers*, W. B. Saunders Co.)

scrape the crest of the fold with oily scalpel blade until lymph begins to ooze. Avoid drawing gross blood.

5. Transfer the scraping from the scalpel to the drop of oil on the slide.

6. Apply a coverglass to the drop with the aid of forceps.

7. Systematically examine the material under the coverglass, using the low power of the microscope and rather low illumination. The oil renders the skin scales transparent and parasites appear rather prominently. Mites may live for several days in such a preparation. In some cases, several scrapings may be necessary in order to find them.

8. Certain of the larger mites may be seen upon gross examination by scraping the lesions with a dry scalpel or knife blade and placing the scraping upon a piece of black paper or cloth exposed to sunlight and warmth. The mites may be seen as tiny white dots moving about. This method, of course, cannot be depended upon for an accurate diagnosis.

Aids to the Species Identification of the Mites.—*Sarcoptes scabiei*, the itch mite. This parasite produces scabies in man and mange in animals. The female Scabiei enters into the skin, producing burrows in which she lays from 15 to 50 eggs. These burrows measure 1 to 10 mm. in length. The usual location for the infestation is the thin skin between the fingers and toes, and about the inguinal and genital regions. The eggs measure about 14 micra in length.

In scrapings from the skin the adult female will be readily seen as a small oval mite, 400 micra in length. The male is much smaller and is similar to the female in shape. The head, thorax and abdomen are fused. There are four pair of legs. The parasite is without eyes. See Figure 291.

Fig. 292.—Female Grain Itch Mite. ×300

(From Schamberg, *Diseases of the Skin and Eruptive Fevers*, W. B. Saunders Co.)

Trombicula irritans, harvest mite, red bug or chiggers. This mite produces a red itching wheal, usually with minute red spot in the center. The parasite penetrates the skin and feeds on the blood of the host. The mite is reddish, oval in shape, and has four pair of legs, a hairy body and pedunculated eyes. In Japan a similar species, *T. akamushi*, is important in the spread of a typhus-like disease known as "Japanese river fever," tsutsugamushi. *T. holosericeum* is the harvest mite of Europe.

Liponyssus bacota is of importance in the tropics, being partly responsible for the spread of typhus from rat to rat.

Pendiculoides ventriculosus is associated with wheat straw, producing skin lesions and at times irregular fever among the individuals coming in contact with the infested straw. The grain or straw itch mite producing acarodermatitis urticarioides (Schamberg) is shown in Figure 292.

Glyphagus domesticus is the food mite, commonly found in sugar, producing in individuals handling such infested food "grocers' itch."

IDENTIFICATION OF TICKS

There are a number of species of ticks which are pests that infest mammals, birds and men. They are primarily of importance as carriers, but the wounds produced by their bites may become secondarily infected with bacteria. They are 1 to 4 mm. in length, flat and oval, tending to taper toward the anterior end, with

no division between the cephalothorax and abdomen. They engorge themselves with blood through a heavily armatured piercing-organ, the hypostome.

Ornithrodorus Moubata.—This is an oval, yellowish-brown tick. It is found on mammals as well as birds. and is important as a vector of West African relapsing fever in man.

Dermacentor Andersoni, Wood Tick.—This is reddish-brown in color, oval, 6 mm. in length. The male shows white and black markings. It is found in the western part of the United States. It infests domestic and wild animals, but is an important vector in the transmission of Rocky Mountain spotted fever and tularemia.

IDENTIFICATION OF TONGUE WORM

Linguatula Serrata, the Common Tongue Worm.—The larvae of this arachnoid is occasionally found in man. The adult female measures 10 cm. in length, and usually occurs in the nasal passages and frontal sinuses of carnivora, occasionally horses and sheep.

The adults rarely infest man. Man acquires the infestation by swallowing the ova from infested animal secretions. The ova hatch out in the intestine, and the larvae eventually reach the liver or nasal cavity and sinuses of man. The larvae are about 5 mm. in length, slightly flattened tongue shape and tapering at the end.

IDENTIFICATION OF FLEAS

A number of species are present in birds and mammals. They are important as vectors of disease. Only one species (Tunga persitans) infests the skin of man.

Rat Flea.—This is important as vector of bubonic plague. from rat to rat and from rat to man. The fleas act as an intermediate host and vector for the dog tapeworm. *Dipylidium craninum.*

Tunga Persitans.—The female flea burrows into the skin of animals and birds and occasionally man. The infestation is confined to the topics.

IDENTIFICATION OF LICE

This group of biting and sucking parasites is probably important as vectors of relapsing fever, trench fever and typhus fever. Three important varieties are found in man. The ova when laid are attached to hairs or clothing, and are spoken of as "nits." Examination of the nit under the microscope will reveal an oval structure with an operculated end, the blunt end being attached to the hair. The eggs hatch in from 7 to 10 days.

Pediculosis Capitis, the Head Louse.—This is found in the hair of the head. The size is from 1 to 2 mm. in length. and nits 0.6 mm. The diagnosis is made by finding the parasites or examining the hair for the nits. The infestation is spread through combs. brushes, hats, etc. For morphology see Figure 293.

Pediculosis Corporis, the Body Louse.—It measures from 2 to 4 mm. in length, the nits 0.8 mm. It attaches itself to the underclothing, particularly the seams. The infestation is spread through interchange of wearing apparel. This

parasite is of importance as a vector of disease. For morphology see Figure 291.

Pediculosis Pubis, the "Crab" Louse.—It receives its name because of the claw-like extremities. It measures 0.8 to 1.2 mm. in length. It selects the hairy portion of the body about the genitalia. The ova are attached at the base of the
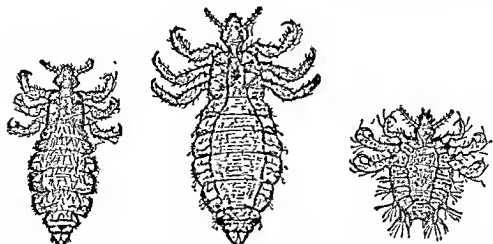
FIG. 293.—PEDICULUS CAPITIS   FIG. 294.—PEDICULUS CORPORIS   FIG. 295.—PEDICULUS PUBIS

(From Schamberg, *Diseases of the Skin and Eruptive Fevers,* W. B. Saunders Co.)

hairs and measure 0.8 mm. The louse is usually transmitted by direct contact. Its morphology is shown in Figure 295.

IDENTIFICATION OF FLIES

Many species of flies are of medical interest. A large number are important as vectors of disease; others are important because of their bite.

In the skin the infestations are limited to the larval forms. At least two varieties actually burrow into the skin, producing tissue-destruction resembling a boil. Other species deposit eggs in open wounds or in the various cavities of the body.

The infestation of fly larvae into the skin is known as cutaneous myiasis. The chief species responsible for wound myiasis are:

The green bottle fly, *Lucilia caesar.*

The common blow fly, *Calliphora vomitoria.*

The meat or flesh flies, *Sarcophaga carnara* and group.

The American screw worm, *Cochliomyia macellaria* (*Chrysomyia macellaria*) and rarely others.

True Cutaneous Myiasis.—*Dermatobia hominis,* a fly indigenous to tropical America. The adult is a little over 1 cm. in length. It has a bluish-green abdomen and dull brownish wings.

The method of transmission to man is not well understood. The larvae bore their way through the skin, and in the subcutaneous tissues produce a lesion resembling a boil.

The diagnosis is made by finding the larvae upon incising the boil. When full grown the larva is 12 mm. in length. The head end is armed with a number of spines. It tapers at the posterior end.

Cordylobia anthrophaga, the tumbu fly, indigenous to Africa. The adult is a yellowish color with black markings. The living larvae are deposited directly on the skin of animals or man. This they penetrate producing a boil-like lesion. When full grown, the larvae are about 1 cm. in length, yellowish-white in color, with an anterior pointed end provided with spines and a rather blunt posterior portion.

The diagnosis is made by demonstration of the larvae upon incising the lesion.

METHODS FOR IDENTIFICATION OF PROTOZOA FOUND IN THE MOUTH

Endamoeba Gingivalis.—This ameba has a wide geographical distribution, and is frequently found about the teeth, particularly those undergoing caries, and in tartar accumulations.

The organism is similar in morphology to the *Endamoeba histolytica*. It measures from 10 to 20 micra. It has well-marked motility. The pseudopodes are short and rather blunt. The ectoplasm is distinct. The organism ingests cell-fragments from food, and may even ingest red cells. The nucleus is much like that of the *Endamoeba histolytica*. See Figure 269.

Method of Examination.—Scrapings from about the teeth, bits of tartar are selected, and examined for the trophozoite (motile form) by direct stained and unstained method.

Trichomonas Buccalis (Trichomonas Elongata).—This flagellate is occasionally met with in material taken from about the teeth, particularly the tartar. It is practically identical in morphology with the *Trichomonas intestinalis*, and is thought by some to be the same species.

Method of Examination.—Material from about the gums or teeth, especially tartar, is suitable for examination, and may be examined by direct stained and unstained methods as given under Amoeba.

METHODS FOR THE IDENTIFICATION OF PROTOZOA FOUND IN THE VAGINA

Trichomonas Vaginalis.—This organism is quite frequently found in vaginal secretions or exudates which are acid in reaction, but are probably not pathogenic. They are similar or identical in structure with the *Trichomonas intestinalis*. See page 262. They measure 15 to 25 micra long and 7 to 12 micra wide.

Method of Examination.—The vaginal secretions or exudate are examined by direct stained or unstained methods for the motile forms as under Amoeba.

URINARY MYIASIS

Urinary myiasis has been described, but is extremely rare. It is due to some groups of diptera.

CHAPTER XXII

METHODS FOR THE PREPARATION OF BACTERIAL VACCINES AND BACTERIOPHAGE

In this chapter are described methods for the preparation of such autogenous and stock bacterial vaccines or bacterins as may be required of a clinical laboratory. The tuberculins, diphtheria and tetanus toxoids, scarlet fever toxin, etc., may be obtained commercially and are omitted.

Preparation of Cultures.—1. Freshly isolated organisms are preferred.

2. The method employed for making cultures is very important in order to secure the organism or organisms responsible for infection. Faulty methods may result in securing only saprophytes or contaminating organisms and defeat the purpose of vaccine therapy at the outset. Methods are described in Chapter XV.

3. It is particularly important to use the proper medium and especially when infection with fastidious organisms is suspected (streptococci, pneumococci, gonococci, etc.). Blood agar or glucose hormone broth are recommended for routine use. The preliminary examination of a stained smear of pus or other material is advised and aids in choosing the proper medium.

4. Incubate cultures for 24 to 48 hours; examine smears stained by the Gram method.

5. If more than one organism is present, secure each in pure culture by plating. While the vaccine is being prepared, subcultures of each may be subjected to final identification.

6. When an organism shows both smooth and rough colonies, select the former for the vaccine.

7. Cultivate the organism or organisms on slants of solid medium until sufficient growths are secured; or cultivate in a broth medium and centrifuge thoroughly. Discard the supernatant fluid and use the sediment of bacteria.

8. Examine each culture by stained smear for purity.

Selection of Organisms.—1. In mixed infections with two or more organisms a selection must be made of those to be incorporated in autogenous vaccines. Do not use spore-forming bacilli (like *B. subtilis*) or saprophytes (like *B. prodigiosus*, diphtheroids, etc.). Organisms of secondary infection may be included.

2. Intracutaneous tests with individual vaccines of each organism are sometimes employed for aid in selection on the principle that only those yielding positive allergic reactions should be employed as indicative of infection (see Chapter XXXI). Great care is required since skin reactions may be purely inflammatory and nonspecific, or due to the presence of toxins for which there are insufficient

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amounts of antitoxin in the blood. The exact value or status of the method is as yet unknown.

The Heist-Cohen Pathogen-Selective Method.—This method may be helpful on the basis that only those organisms capable of surviving and growing in the whole coagulated blood of the patient are apt to be infective. It is possible, however, that an organism unable to grow in the blood or produce septicemia may still be able to produce local infection.

1. Secure material to be cultured on sterile swabs (from nose, tonsils, extracted teeth, tooth sockets, sputum, pus, etc.), and rub each swab on the bottom of dry sterile tubes into which one or two drops of broth have been placed.

2. Then place the swabs into tubes of hormone broth medium for controls and also for use in preparing the vaccine.
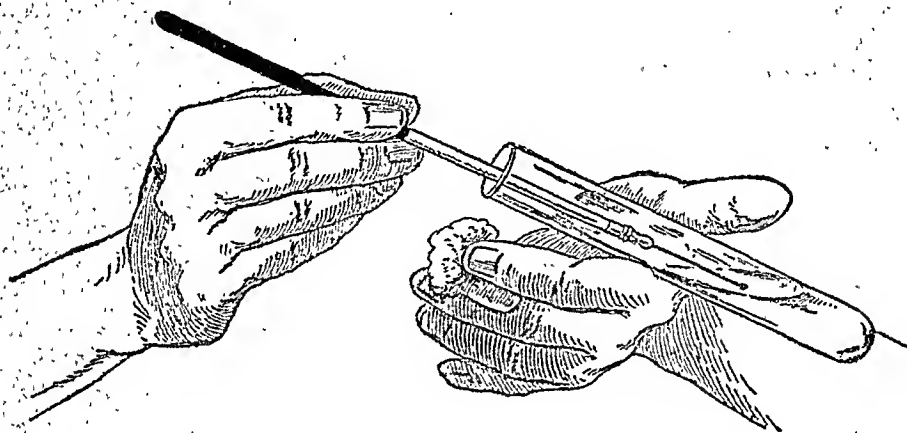
FIG. 296.—PREPARATION OF A BACTERIAL VACCINE

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

3. Immediately secure from 10 to 12 c.c. of blood from a vein at the elbow of the patient with a sterile syringe and with aseptic precautions.

4. Place 3 to 5 c.c. of blood in each tube in which material has been smeared.

5. Incubate the blood and the control tubes for 24 hours. Examine each by smear. If necessary, plate each on blood agar for identification of organisms. Also inoculate a tube of hormone broth from each blood tube showing a growth as a check on the plate and for preparing the vaccine.

6. The vaccine is now made up by mixing 1 part of the original broth culture and 9 parts of the broth subculture of the blood.

7. Count the mixed suspension and finish up the vaccine according to steps 3, 4, 5 and 6 of the Kolmer method described on page 549.

PREPARATION OF SUSPENSIONS

1. If a solid medium is used, cover the growth on one of the tubes with sterile salt solution, taking precautions against contamination. Bring the organism into suspension either by shaking or with a platinum loop (Fig. 296).

(same as above) to 10] to give a 1 : 200 dilution. After thorough agitation, discard a few drops and place a drop into the counting cell. Adjust the coverglass and allow 15 minutes for thorough settling. Make a count of 10 to 20 squares with the following formula:

$$\frac{\text{total bacteria counted} \times 200 \times 20{,}000{,}000}{\text{number of small squares counted}} = \text{bacteria per c.c.}$$

14. Place the counting chambers and cover slides in 2% cresol for at least 5 minutes before wiping. Clean the pipets in the same before drying.

15. After counting, the vaccine is sterilized and diluted to proper strength as described on page 517.

Wright's Method.—1. Make a mark on the stem of a capillary pipet about one inch from the tip and fit a rubber bulb to its barrel.

2. Cleanse and prick the finger.

3. Draw up into the capillary pipet sodium citrate solution to the mark on the tip. Then draw a little air in, then blood from the finger up to the mark. Draw a little air in to separate the solution, next draw bacterial suspension up to the mark (Fig. 299).

4. Expel the contents of the pipet on a glass slide or in a watch glass and mix thoroughly by aspirating and re-expelling about a dozen times (Fig. 300).

5. Make two or three thin films on slides in the same manner as described for blood smears (Fig. 301).

6. Dry in air and fix with a saturated solution of corrosive sublimate.

7. Wash and stain with dilute carbolfuchsin (1:10) or carbolthionin for 2 to 5 minutes.

8. Wash and dry.

9. Examine only satisfactory films which show bacteria and blood cells in approximately the same numbers and free from bacterial aggregate (Fig. 302). With oil-immersion lens count the number of corpuscles and bacteria in a number of fields, or until 500 corpuscles have been counted. Mark down the number of cells and bacteria counted separately and total each at the end.

10. Calculation: Let us assume that 500 red cells and 1000 bacteria have been counted. One c.mm. of blood contains approximately 5,000,000 red corpuscles and equal volumes of blood and emulsion were taken. One c.mm. of the emulsion, therefore contains $\dfrac{5{,}000{,}000 \times 1000}{500} = 10{,}000{,}000$ organisms per c.mm., or 10,000,000,000 per c.c.

11. Dilute to proper strength and sterilize (page 547).

FIG. 299.—A CAPILLARY PIPET FOR COUNTING A BACTERIAL VACCINE
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

Bacterial Emulsion | Air | Blood | Air | Sod. Citrate Sol.

Nephelometer Method (McFarland).—1. *This method is mainly applicable for suspensions prepared from agar slants or from centrifuged broth cultures resuspended in saline solution* (Fig. 303).

2. Place 1 c.c. of bacterial suspension in a test tube which should be of the same size as those used in the nephelometer.
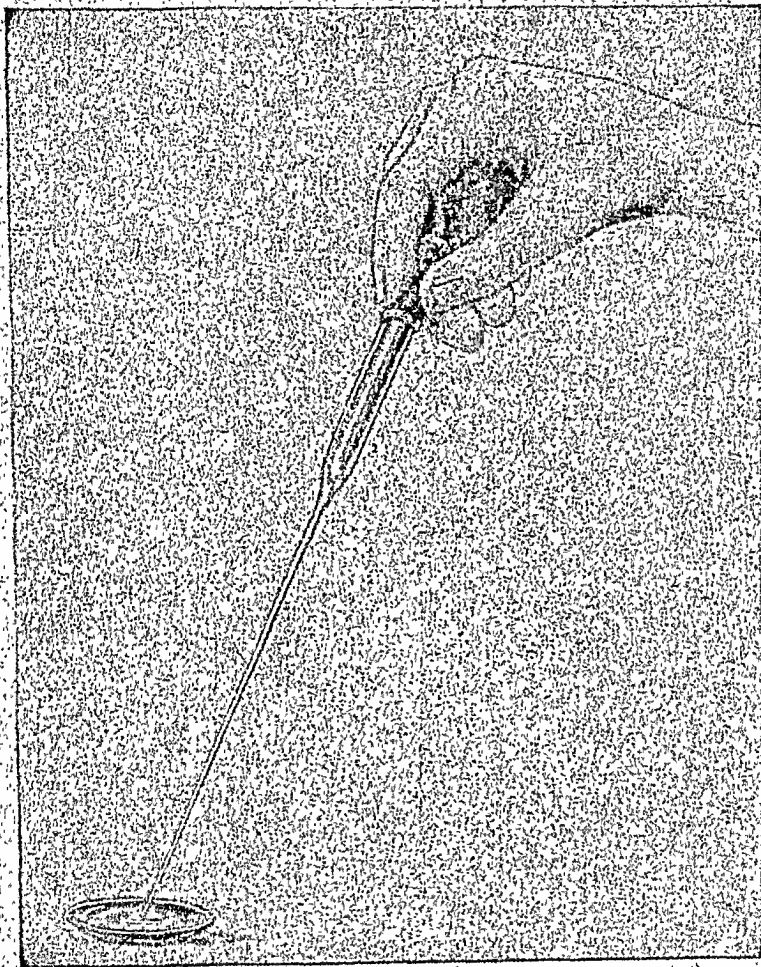
Fig. 300.—Mixing the Contents of a Pipet

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

3. Dilute with 4 to 10 c.c. of salt solution, keeping accurate record of the final dilution.

4. Compare with tubes of nephelometer. Shake the tubes well before comparing.

5. Calculation: Multiply the number of bacteria represented by the nephelometer tube which corresponds with the density of the bacterial suspension by the dilution of the bacterial suspension. For example, assume that the density of the bacterial suspension corresponds to the No. 3 tube of the nephelometer and before

making comparison it was diluted 8 times. No. 3 tube corresponds to 1,000,000,000; 8 times this number equals 8,000,000,000 bacteria per c.c. Or simply dilute vaccine to correspond in density to tubes 3 to 4 of the nephelometer to secure approxi-

FIG. 301.—SMEARS FOR VACCINE COUNTS

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

mately 1,000,000,000 per c.c. for adults; or to correspond to tubes 1 to 2 to secure approximately 500,000,000 per c.c. for children.

FIG. 302.—COUNTING VACCINE

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

6. The nephelometer is prepared as follows:

(a) Arrange 10 test tubes or ampules of uniform size in a rack, and label 1 to 10.

(b) Add the following amounts of a 1% solution of chemically pure barium chloride: To tube No. 1, 0.1 c.c.; tube No. 2, 0.2 c.c.; and so on, increasing 0.1 c.c. in each.

(c) Add sufficient of a 1% chemically pure sulphuric acid solution to make the total volume 10 c.c. in each tube.

(d) Seal the tubes or ampules.

(e) When the fine white precipitate of barium sulphate, which has formed in the tubes, is shaken up well, each tube will have a different density, increasing from Nos. 1 to 10. The density of the tubes corresponds *approximately* to bacterial suspension, as follows:

No. 1:	300,000,000	No. 6: 1,800,000,000
No. 2:	600,000,000	No. 7: 2,100,000,000
No. 3:	900,000,000	No. 8: 2,400,000,000
No. 4: 1,200.000,000		No. 9: 2,700,000,000
No. 5: 1,500,000,000		No. 10: 3,000,000,000

7. If vaccines are prepared of broth cultures, the nephelometer should be prepared with 1% sulphuric acid in broth in order to convey the color of the latter.

DILUTING, STERILIZING AND PRESERVING VACCINES

Chemical Sterilization.—1. Vaccines sterilized with tricresol, cresol, merthiolate or phenol without the aid of heat are commonly regarded as being more antigenic than heat-killed vaccines.
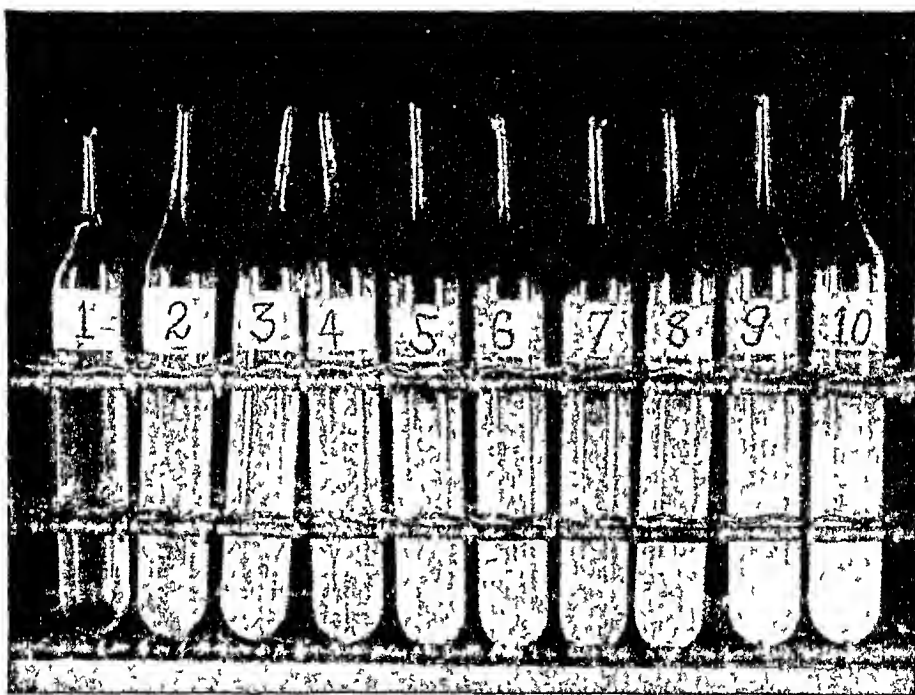
FIG. 303.—NEPHELOMETER

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

2. Tricresol is recommended in a final concentration of 0.5% for sterilization and preservation. A saturated solution (approximately 5%) in water may be employed, adding 1.0 c.c. for each 10 c.c. of vaccine to give the final concentration of 0.5%.

3. Proceed as per the following example: 20 c.c. of vaccine containing 1,000,000,000 per c.c. is desired. The suspension contains 2,700,000,000 per c.c.

$$\frac{1000 \times 20}{2700} = 7.0 \text{ c.c. of suspension to be used}$$

4. In a sterile vial or bottle containing a few glass beads place:·7 c.c. of suspension; 2 c.c. of 5% tricresol; 11 c.c. of sterile saline.

5. If the vaccine is to be a mixed one, prepare separate vaccines of each organism in this manner and then mix equal parts of each.

6. Stopper with a sterile rubber cap, mix and place in the incubator at 37° C. for 24 hours.

7. Remove 0.5 c.c. with a sterile syringe and needle and place in a flask of at least 50 c.c. of a suitable broth medium for sterility test. Incubate 24 to 48 hours and dispense the vaccine as ready for administration if sterile.

8. If not sterile, reculture the vaccine. As a general rule, 24 hours·at 37° C. are sufficient unless spores are present.

Sterilization by Heat.—1. Stopper the vial or bottle with a rubber cap and immerse in a bath of cold water reaching above the level of the vaccine.

2. Place a thermometer in the bath, raise the temperature up to 60° C. and maintain it for one hour.

3. Make a culture of the vaccine for sterility as described above except that a tube of broth may be used.

4. If not sterile, the vaccine may be reheated for another hour although this may reduce its antigenic activity. As a general rule, one hour at 60° C. is sufficient unless contaminating spores are present, in which case it should be discarded.

METHODS FOR DISPENSING VACCINES

1. It is quite convenient to dispense vaccines in vials or small bottles (Fig. 304), stoppered with rubber caps, and labeled with name of organism and number per c.c.

2. The first dose may be of 0.1 c.c. and subsequent doses gradually increased, as by 0.1 or 0.2 c.c. according to reactions. These amounts are readily removed with a suitable syringe and needle after disinfecting the rubber cap with tincture of iodine or some other suitable disinfectant.

3. Some physicians prefer having vaccines dispensed in ampules (Fig. 305).

4. The designated doses, as 0.1, 0.2, 0.3, 0.4, c.c., etc., are placed in small sterile ampules with a sterile pipet and the volume in each brought up to 1 c.c. by adding tricresolized saline solution (0.3 c.c. of trieresol in 100 c.c. of saline). The neck of each ampule is then sealed in a flame and each labeled with a number or the dose.

PREPARATION OF CULTURE FILTRATE VACCINE

1. The soluble exogenous toxins or their toxoids are highly antigenic and sometimes used as vaccine. In autogenous vaccines these refer especially to the toxins of staphylococci and streptococci.

2. Cultivate the organism in broth at 37° C. for at least 4 or 5 days.

3. Filter through a small sterile Berkefeld or Mandler filter.

4. Test the filtrate for sterility by culturing 0.5 c.c. in a tube of broth for at least 24 hours.

FIG. 304.—BOTTLE AND CAP FOR
BACTERIAL VACCINE

(From Kolmer, *Infection, Immunity
and Biologic Therapy*, W. B. Saunders
Co.)
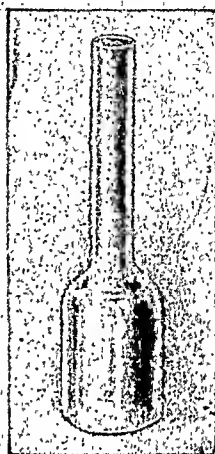
FIG. 305.—A SMALL
VACCINE AMPULE

(From Kolmer,
*Infection, Immunity
and Biologic Ther-
apy*, W. B. Saunders
Co.)

5. Do not heat, as this destroys some toxins.

6. For preservative, add 0.6 c.c. of 5% tricresol to each 10 c.c. of filtrate or the filtrate may be used without a preservative if due precautions are taken against contamination.

7. The first dose for an adult should not exceed 0.05 c.c., as these vaccines produce more local and systemic reactions than ordinary heat-killed vaccines.

8. If necessary, the filtrate may be diluted with tricresolized saline (0.3 c.c. tricresol in 100 c.c. of saline solution).

THE KOLMER METHOD FOR THE PREPARATION OF AUTOGENOUS VACCINES

1. This method is based upon employing any exogenous toxins produced by bacteria as well as the organism themselves.

2. A pure culture of the organism is cultivated at 37° C. for at least 4 to 5 days in a hormone broth medium (do not use serum or blood broth). If there are 2 or more organisms, each is grown separately in pure culture and made up into separate vaccines, which are finally mixed together in equal proportions to give a single vaccine of desired numerical strength.

3. The broth culture is counted by the counting chamber or Wright method described above or estimated by a nephelometer prepared with broth.

4. Proceed as per following example: 20 c.c. of vaccine containing 1,000,000,000 per c.c. are desired. Count or estimate shows 5,200,000,000 per c.c.

$$\frac{1000 \times 20}{5200} = 3.6 \text{ c.c. of suspension to use}$$

5. In a sterile vial or bottle containing a few glass beads place: 3.6 c.c. of broth suspension; 2.0 c.c. of 5% tricresol; 14.4 c.c. of sterile saline.

6. If the vaccine is to be a mixed one prepare separate vaccines of each organism in this manner and then mix equal parts of each.

7. Stopper with a sterile rubber cap, mix and incubate at 37° C. for 24 hours. Do not heat at 55° to 60° C. as this destroys thermolabile toxins and particularly those produced by staphylococci.

8. Culture 0.2 c.c. in at least 50 c.c. of a suitable broth medium for sterility.

9. If sterile, the vaccine is ready for administration.

10. Vaccines prepared by this method are of a light brown color and may give slightly more local reaction at the site of infection. The first dose may be 0.1 or 0.2 c.c. and subsequent doses slightly increased.

PREPARATION OF TYPHOID-PARATYPHOID VACCINE

1. Use the Panama carrier strain of *B. typhosus* (National Institute of Health No. 58); the kessel strain of *B. paratyphosus A* and the Rowland strain of *B. paratyphosus B*. These may be obtained from the Army Medical School, Washington, D. C.

2. Culture each strain in a tube of broth and examine for purity.

3. Inoculate Blake bottles of agar with each strain if a large amount of vaccine is to be prepared; for smaller amounts inoculate 24 slants of agar with *B. typhosus*, 24 with *B. paratyphosus A* and 24 with *B. paratyphosus B*.

4. Incubate for 2 to 3 days and examine for purity.

5. Prepare separate *heavy* suspensions of the 3 organisms by washing off the agar cultures with appropriate amounts of sterile saline solution.

6. Shake each suspension with sterile glass beads to break up clumps and filter each through sterile paper.

7. Count each suspension by the counting chamber method described above.

8. The finished vaccine should contain in each c.c.:

> 1,000,000,000 *B. typhosus*
> 750,000,000 *B. paratyphosus A*
> 750,000,000 *B. paratyphosus B*

9. Proceed as per the following example: It is desired to make 500 c.c. of finished triple vaccine. Count of typhoid suspension is 3,200,000,000 per c.c. Count of para A suspension is 6,500,000,000 per c.c. Count of para B suspension is 7,900,000,000 per c.c.

$$\frac{1000 \times 500}{8200} = 61 \text{ c.c. of typhoid suspension to be used}$$

$$\frac{750 \times 500}{6500} = 57.7 \text{ c.c. of para A suspension to be used}$$

$$\frac{750 \times 500}{7900} = 47.5 \text{ c.c. of para B suspension to be used}$$

10. In a sterile bottle place: 61 c.c. of typhoid suspension; 57.7 c.c. of para A suspension; 47.5 c.c. of para B suspension; 75 c.c. of 2% solution of tricresol; 258.8 c.c. of sterile saline solution.

11. Mix well. This gives 500 c.c. of triple vaccine of the desired strength of each organism preserved with 0.3% tricresol.

12. Place the bottle in a bath of cold water reaching above the level of the vaccine.

13. Place a thermometer and heat at 53° C. for one hour.

14. Culture 1 c.c. in a small flask of broth for sterility: incubate for 48 hours. Make anaerobic culture and inoculate a rabbit and mouse for additional tests for sterility.

15. For adults the doses at weekly intervals are as follows by subcutaneous injection: 0.5, 1.0 and 1.0 c.c.

PREPARATION OF UNDENATURED VACCINES (KRUEGER)

Denaturation of bacterial proteins by heat or chemical changes in the preparation of vaccines may result in a reduction of antigenic activity. To prevent these changes Krueger[1] has devised a method for fragmentation of bacterial cells and putting into solution or suspension the cellular components. The technic is somewhat too complicated for the average clinical laboratory; the principles are as follows (consult reference for technical details):

Mass cultures of bacteria are grown on Blake flasks on appropriate media. The cells are harvested in buffered isotonic solution and thoroughly washed free of metabolites. After the final centrifugation the bacteria are suspended in the buffer solution and the cell count made. The dense suspension is placed in a special type of ball mill and mechanically disrupted over a period of 10 to 18 hours. Residual cells are removed by ultrafiltration through acetic collodion membranes of such porosity that all intact bacterial cells are held back while material in solution or in a finely dispersed phase passes through without substantial reduction in concentration, such as occurs when filter candles are employed (adsorption). The water-clear filtrate contains those constituents of the live bacterial cell put into solution or suspension by physical rupture of the cell membrane. There have been excluded immunologically undesirable constituents, such as metabolic products and degradation products resulting from the heat or chemical treatment of the bacteria employed in orthodox methods of vaccine preparation. The final solution is standardized on the basis of its nitrogen and protein content.

[1] J. Infect. Dis., 1933, 53:237.

ISOLATION AND PREPARATION OF BACTERIOPHAGE

Filters.—1. The Berkefeld 3W and 5W are recommended, the 5W filter candles for small volumes and the 3W for larger amounts. The test tube containing the filtrate may be replaced by an empty sterile tube and the filter used 2 or 3 times for succeeding generations if the material is not too cloudy.

2. When the new filters are received the chalky deposit is removed from the outside by scrubbing gently under running water with a fine brush (nail brush) which has not been used with soap. Then the filters are boiled 15 to 20 minutes
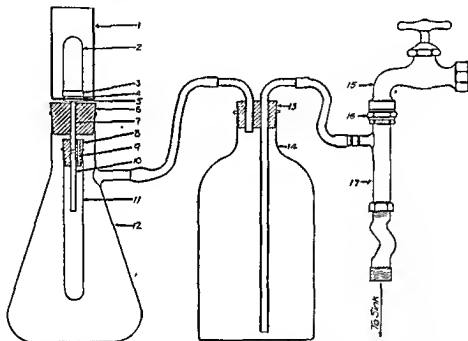
Fig. 306.—Method of Filtration for the Preparation of Bacteriophage.

(1) mantle; (2, 3, 4, 5) filter; (6) perforated rubber stopper; (7) neck of filter; (8) perforated rubber stopper in test tube (11); (12) filter flask with side-arm; (13) perforated rubber stopper in air-exhaustion bottle (14); (15) faucet; (16) connection for suction pump (17).

in distilled water 3 times. This tends to neutralize the filters and to cleanse them. The mantles are washed with soap and water and allowed to drain until they are dry.

3. They are assembled according to Figure 306. For ordinary set-ups a regular test tube holding 15 to 20 c.c. is used but in preparing a large volume a larger test tube holding about 60 c.c. is used. The air is drawn out of the Erlenmeyer flask by the use of suction and then out of the test tube through the second hole and between the two corks producing sufficient suction for filtration. The airway is so narrow and well protected that the filters when assembled

and sterilized may be kept without being wrapped for several weeks before being used.

4. After being assembled the filters are sterilized in a steam autoclave at 121° C. for 1 hour.

5. After use the filters are dissembled and the 3 parts, the candles, mantles, and corks are boiled separately in tap-water for 20 minutes to disinfect them. If necessary they are autoclaved before being dissembled. After the boiling the filters are scrubbed and boiled as when new, the mantles are washed with soap and water and allowed to dry and the corks are rinsed. Then they are again ready to be assembled.

6. About every fifth time they are used before the three boilings in distilled water the candles are attached to the suction pump and distilled water is run through them for a few minutes to cleanse the interior.

Method for Isolation of Bacteriophage from Feces.—1. Disintegrate thoroughly by suspending about 5 grams in 50 c.c. of broth (pH 7.4 to 7.8) and incubating 12 to 24 hours at 37° C.

2. Then centrifuge to remove large particles and filter through infusorial earth: provide a funnel with a folded filter paper large enough to receive at one time the entire volume to be filtered. Fill the filter with water to which has been added a small amount of infusorial earth. When the water has passed through, the paper is left coated with a thin layer of the infusorial earth, thus rendering the paper less permeable.

3. Then pass the filtrate through a sterile Berkefeld or similar filter. This removes the bacteria and the filtrate should be tested for bacteriophage for *B. coli*, *B. dysenteriae*, *B. typhosus*, staphylococci, streptococci, etc., as detailed in the descriptions given below.

Method for Isolation of Bacteriophage from Sludge and Sewage.—1. These are the most common sources and a bacteriophage for the colon bacillus can be almost invariably obtained.

2. Filter through paper or fine gauze to remove large particles.

3. Pass filtrate through a sterile Berkefeld filter. Culture 1 c.c. in broth for 48 hours for sterility.

4. Or the following method may be employed: Inoculate a flask of broth with 10 c.c. of sewage. Incubate over night. Pass through an infusorial earth filter and then through a sterile Berkefeld to remove bacteria. Inoculate tubes carrying 20 c.c. of double strength broth (pH 7.4 to 7.8) with 5 or 10 c.c. of filtrate. Incubate 24 hours and filter through a sterile Berkefeld filter. The filtrate is then tested for bacteriophage.

Method for Testing Feces and Sewage Filtrates for Bacteriophage.—1. Prepare a suspension of the test organism from 18 to 24 hour agar slant culture of such density that 0.1 c.c. added to 10 c.c. of broth will give a faint perceptible cloud.

2. To each of 4 tubes carrying 10 c.c. of broth (pH 7.6-7.8) add 0.1 c.c. of the suspension of test organism. To 3 of the tubes add 0.5, 1, and 2 c.c. of the filtrate

to be tested for bacteriophage. The fourth tube is a culture control. To a fifth tube of 10 c.c. of broth add 0.5 c.c. of filtrate as a control on its sterility.

3. Incubate at 37° C. until there is a perceptible growth in the control (usually 4 hours). Observe carefully for clearing (lysis): $+ + + + =$ complete clearing; $+ + + + =$ slightly cloudy; no sediment; $+ + =$ perceptible clearing; some sediment; $+ =$ slightly clearer than the control.

4. Filter the first 2 tubes through sterile Berkefelds. Continue incubation of the third tube overnight. A reading is then made using the same scale. If lysis is not $+ + + +$, the process is repeated, using the filtrate obtained after the 4-hour incubation. When lysis is complete, a volume of phage is prepared, using the same materials and amounts, but setting up a large number of tubes.

Method of Isolating Staphylococcus Bacteriophage from Pus.—1. Place 1 c.c. of pus in 20 c.c. of broth or 5 c.c. in a flask of broth (pH 7.6-7.8) and incubate at 37° C. overnight.

2. Filter through sterile sand, then sterile paper and finally a sterile Berkefeld filter (pus filtrate).

3. Prepare a thin suspension of the staphylococcus in broth.

4. In each of 5 sterile test tubes place 10 c.c. of broth (pH 7.6-7.8).

5. Add 0.1 c.c. of the bacterial suspension to the first four tubes.

6. To the first three add 0.5, 1 and 2 c.c. of the pus filtrate respectively. Tube No. 4 is a culture control. To No. 5 add 0.5 c.c. of pus filtrate as a control.

7. Incubate 4 to 2½ hours and examine frequently. If bacteriophage is present lysis will occur in some or all of the first three tubes. No. 4 should show a good growth. No. 5 should be sterile and remain clear.

Method for Testing and Dispensing Bacteriophage.—1. Bacteriophage should not be used in treatment unless first shown to be lytic for the organism producing the infection. Commercial and stock phages should always be tested for lysis for the infecting organism before used.

2. Cultivate the organism in plain or blood agar for 18 to 24 hours and prepare a suspension in broth. If the growth is scant, use broth.

3. Place 9.5 c.c. of broth or asparagin medium in each of 2 test tubes. To both add 0.1 c.c. of the culture suspension sufficient to give a barely perceptible turbidity. To tube No. 1 add 0.5 c.c. of the bacteriophage being tested.

4. Incubate both tubes at 37° C.; with staphylococcus or B. coli for 4 hours; with streptococcus about 6 to 12 hours.

5. Examine the first tube for lysis. The second or culture control tube should show a growth with increased turbidity.

6. If an asparagin bacteriophage is desired for intravenous use, it is advisable to carry it for two generations in asparagin medium, thus reducing the broth concentration to a negligible minimum.

7. Bacteriophage should be cultured by placing 1 c.c. in broth and incubating 48 hours. None should be used which shows the slightest growth.

8. Very careful aseptic precautions should be exercised in dispensing. A preservative is not advisable as it may inhibit bacteriophage.

9. A bacteriophage made up with an organism from a patient's culture is called "autogenous." Stock bacteriophage is recommended in acute cases until the specific is prepared.

10. Bacteriophages should be kept in a refrigerator where they maintain potency for 4 months to 3 years.

Stock Bacteriophages.—1. These should be polyvalent and of high titer.

2. Stock staphylococcus and *B. coli* bacteriophages are most widely employed.

3. Streptococcus bacteriophage is highly specific and $++++$ lysis is seldom obtained. Bacteriophage for hemolytic streptococci are prepared much more readily than for nonhemolytic types including *Streptococcus viridans*. Partially potent phages are sometimes used and have been found of some value in treatment.

4. In mixed infections, the bacteriophages are mixed in proportions indicated by the relative numbers of organisms in cultures.

D'Herelle's Method for Titrating the Potency of Bacteriophage.—1. To 60 c.c. of broth add 0.2 c.c. of an 18- to 24-hour broth culture of organism (bacterial suspension).

2. Set up 12 sterile test tubes and place 4.5 c.c. of the bacterial suspension in each.

3. To No. 1 add 0.5 c.c. of bacteriophage.

4. Mix well and transfer 0.5 c.c. to No. 2; mix well and transfer 0.5 c.c. to No. 3 and so on to No. 12 from which discard 0.5 c.c. Use a fresh sterile pipet for each transfer.

5. Include a control of 5 c.c. with bacterial suspension.

6. Incubate 24 to 48 hours and examine for lysis.

Method for Preparing B. Coli and Staphylococcus Bacteriophage for Therapy (New York Post-Graduate Hospital).—1. The medium is plain beef infusion broth adjusted to pH 7.4 to 7.8. If for subcutaneous or intravenous administration and in case of protein allergy, an asparagin medium is preferred prepared as follows:

Asparagin (Merck)	3	gms.
Magnesium sulphate	2	gms.
Sodium chloride	4.5	gms.
Bipotassium hydrogen phosphate	2	gms.
Distilled water (neutral)	1000	c.c.

Dissolve in the water; bring to a boil; adjust to pH 7.6; autoclave at 121° C. for 15 minutes; filter through double paper; tube; autoclave a second time at 15 pounds for 30 minutes (final pH 7.0-7.2); should be clear.

2. Two pure cultures of each organism isolated from the case should be prepared on agar slants. One culture is to be kept in ice-box, while phage is being prepared, in case the other becomes contaminated.

3. Use 18- to 24-hour old agar slant cultures. Cover slant with broth making bacterial suspension. If the organism is *B. coli* set up 3 tubes of broth:

4. To all 3 tubes add 0.1 c.c. of a light suspension of the case organism, enough to produce a just perceptible cloudiness.

5. To each of 2 tubes then add 0.5 c.c. of mixed coli phage and label first generation. The third tube receives no phage but serves as a control. Shake and incubate at from 32° C. to 37° C. After 4 to 5 hours there should be a good growth in control tube. Take readings of phage tubes by comparing cloudiness with control:

0—No lysis—tube with phage not clearer than control.

±—Possible lysis—tube with phage possibly clearer than control.

1+—Little lysis—tube with phage definitely clearer than control.

2+—Semi lysis—tube with phage halfway between clear and control.

3+—Good lysis—tube with phage ¾ between clear and control.

4+—Complete lysis—tube with phage crystal clear.

1+ and 2+—May have sediment at bottom.

3+ and 4+—Have no sediment at bottom.

6. Whether there is lysis or not, filter one tube at this time. Put the other tube back in incubator for later readings. Filtrates should be tested for sterility.

7. The following day set up second generation in the same way, using 0.5 c.c. of first generation filtrate instead of phage mixture, using fresh 24-hour old culture of organism made the day before. By successive generations and filtrations, the potency of a phage may be increased. The aim is to obtain 4+ (clear) lysis for 24 hours' incubation or longer. This is not always possible but frequently is accomplished.

8. Staphylococcus bacteriophage is prepared in the same manner except that 0.5 c.c. staphylococcus phage mixture is used instead of coli phage. Complete lysis is obtained in the majority of cases from stock phage in the first generation with this organism. If organism is resistant, it is not usually possible to prepare a phage by successive filtrations.

9. If the organism is susceptible a volume of phage for therapy may be obtained by setting up 6 or more tubes and a control in the usual way and filtering into a larger sized tube, or larger amounts may be prepared by using 250 c.c. of broth with corresponding amounts of culture and stock phage.

Method for Preparing Streptococcus Bacteriophage for Therapy (New York Post-Graduate Hospital).—1. The preparation of streptococcus bacteriophage presents much more difficulty.

2. The transplant of the organism is generally carried along in plain broth. A 24-hour growth is employed in the test for susceptibility to bacteriophage.

3. Five sterile test tubes, 3 of which contain approximately 10 c.c. of plain broth are employed. If the phage is for subcutaneous or intravenous administration, and in case of protein allergy, the asparagin medium previously described is preferred. To each of the 2 empty tubes 10 c.c. of a mixed streptococcus bacteriophage are added. To the third, fourth and fifth tubes, each of which contains broth, are added respectively 2 c.c., 1 c.c., and 0.5 c.c. of the bacteriophage.

4. To each of these 5 tubes and to 1 tube containing only broth is added 0.1 c.c. of the 24-hour growth of the streptococcus to be tested.

5. All tubes are shaken well and incubated for 18 hours at 32° C. to 37° C. A reading is taken at this time using the tube with no bacteriophage in it as a control.

6. Then the 5 tubes containing bacteriophage are filtered through a Berkefeld filter pouring off the top and leaving the sediment at the bottom.

7. This filtrate is the filtrate of the first generation and is used to set up the second generation in the same way, using the filtrate in place of the phage. Ordinarily one finds a partial lysis in the whole filtrate in the second generation. It is usually possible to enhance the bacteriophage by these serial filtrations so that it will produce a 4 + lysis in the whole filtrate and a partial lysis in the tubes with small amounts of filtrate.

8. Occasionally after serial filtration the phage will adapt itself so that a small amount will give complete lysis. Then it is possible to prepare large quantities as in working with the *B. coli* and staphylococcus. Ordinarily, however, it is necessary to repeat the set-ups and filter gaining only small volumes at a time.

CHAPTER XXIII

METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF MILK

Principles.—1. The methods here given are the standard methods of milk analysis of the American Public Health Association and the Association of Official Agricultural Chemists. They are given herewith for guiding the examination of milk in clinical laboratories, especially those connected with hospitals.

2. The total bacterial count continues to be of most value in the bacteriological examination of milk and is especially useful as a measure of the care with which milk is collected and kept until used.

3. Methods for the detection of tubercle bacilli in milk are described on page 482. Unfortunately, however, there are no thoroughly reliable methods at present for the detection of typhoid and dysentery bacilli, *Br. abortus*, and other pathogenic organisms known to be sometimes transmitted by milk. It is still necessary to rely mainly for the elimination of these upon thorough pasteurization, veterinary inspection of herds, and the medical examination and supervision of dairy employees with special reference to typhoid carriers.

4. It is true, however, that the presence of mastitis in cows is sometimes to be detected by finding exceedingly large numbers of long-chained streptococci and pus cells when due care is taken to examine the milk within 6 hours after collection or when carefully refrigerated to prevent multiplication. Due care, however, must be exercised in the examination of sediments secured by centrifuging against mistaking normal streptococci and those contained in butter starters, or derived from dirty milking machine tubes, for pathogenic streptococci.

STANDARD PLATE METHOD FOR TOTAL BACTERIAL COUNTS

1. If bottled milk is to be examined, the bottle should be immediately iced. In case the milk is in bulk, a sample should be taken after thorough mixing. A sterile glass tube long enough to reach from the top to the bottom of the container to be sampled is very satisfactory. The tube is lowered to the bottom and the end closed with the thumb or a finger to hold the contents in the tube, which is then placed in a sample bottle. The sample bottle should be sterile and large enough to hold the entire amount in the tube. Sample bottles should be glass stoppered as cotton plugs are not satisfactory. *Do not collect less than 10 c.c.*

2. If the sample is not to be examined immediately, place it in cracked ice so as to cool promptly to near the freezing point. Prepare dilution bottles to contain 99 c.c. of water after sterilization in the autoclave. They should have rubber or

glass stoppers. The number of bottles will depend upon the number of samples to be examined and dilutions desired.

3. Shake sample 25 times, each shake being an up and down excursion of about 1 foot. Then immediately transfer 1 c.c. to dilution bottle No. 1 (this makes a dilution of 1:100).

4. Shake dilution No. 1 25 times and transfer 1 c.c. to dilution bottle No. 2. At the same time transfer 1 c.c. and 0.1 c.c. to 2 empty sterile Petri dishes. Mark the plates 1:100 and 1:1000.

5. Shake dilution No. 2 and transfer 1 c.c. and 0.1 c.c. to 2 empty Petri dishes and mark them 1:10,000 and 1:100,000. A special pipet is recommended which delivers 1.1 c.c.

6. Melt nutrient beef extract agar with a pH of 6.6 and cool to between 40° and 45° C.

Fig. 307.—Stewart Counting Apparatus

7. Pour 10 c.c. into each plate and mix with the diluted milk by gently rotating.
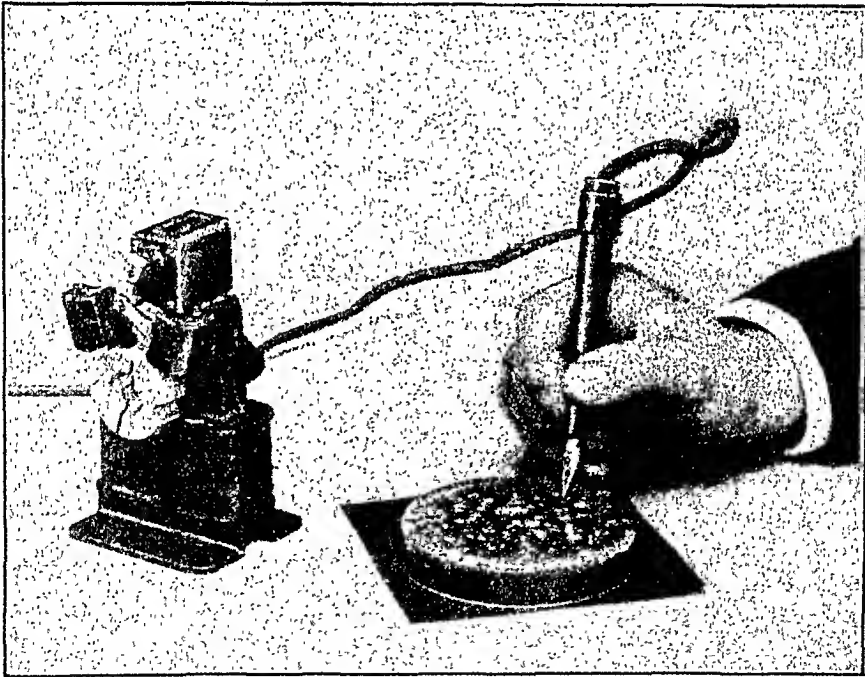
Fig. 308.—Robinson's Colony Counter

Sufficient agar should be used to avoid drying out. An excess will favor the spreading of surface colonies.

8. Incubate all plates for 48 hours at 37° C. in an inverted position.

9. Select plates showing between 30 and 300 colonies. Count the number of colonies on the plates, using a lens magnifying about 2.5 diameters. A colony counting chamber with uniform illumination and with standard ruling should be used (Fig. 307). If the number of colonies exceeds 300, a fraction of the plate can be counted and the number multiplied by the factor and then by the dilution. Plates with less than 20 colonies should not be counted unless no others are available. When many samples are counted daily, Robinson's electric counting device may be found useful and time-saving (Fig. 308). In case of doubt, the compound microscope may be used to distinguish between colonies and debris.

10. Multiply the numbers of colonies by the dilution marked on the plate. This will give the number of bacteria per c.c of milk. Use only two significant left-hand digits in any report. Raise to the next highest round number but never lower.

11. *A series of at least four or more samples should be examined before judging the quality of a given milk supply.*

12. In addition to other requirements, the bacteria counts required before delivery of various grades of milk according to the standard milk ordinance of the United States Public Health Service, are as follows:

> Certified Milk: Less than 10,000 per c.c.
> Grade "A" Raw: Not over 50,000 per c.c.
> Grade "B" Raw: Not over 200,000 per c.c.
> Grade "C" Raw: Not over 1,000,000 per c.c.
> Grade "D" Raw: Not over 5,000,000 per c.c.
> Grade "A" Pasteurized: Not over 10,000 per c.c.
> Grade "B" Pasteurized: Not over 100,000 per c.c.
> Grade "C" Pasteurized: Not over 500,000 per c.c.

DIRECT MICROSCOPIC COUNT OF BACTERIA

Breed Method.—1. The collection of sample is the same as described above for the plate count.

FIG. 309.—BREED AND BREW CAPILLARY PIPET

FIG. 310.—BREED AND BREW GUIDE PLATE

2. Thoroughly shake the sample and deposit 0.01 c.c. of the milk on a clean slide by means of a special pipet (Fig. 309).

3. Spread the milk evenly over an area of 1 square centimeter with a clean stiff needle. The slide can be laid on paper ruled in 1 centimeter squares or on any

ruled guide plate (Fig. 310). This ruling will show the area to be covered by the smear.

4. Dry the film in a warm place. Avoid excess heat as it may cause the film to crack. The drying should be complete within 5 to 10 minutes.

5. Dip the slide in xylol to remove the fat (at least 1 minute). Drain and allow to dry.

6. Place in 90% alcohol for one or more minutes.

7. Transfer to Löffler's methylene blue for five minutes (to overstain).

8. Rinse in water and then decolorize with alcohol. Check the decolorization by observation to avoid overdecolorizing. When properly done the background should show a faint pale blue. If decolorization is carried too far, the smear can be restained.

9. Or the following stain, devised by Newman, may be employed:

Methylene blue (certified powder)	1.12 gm.
Ethyl alcohol (95 per cent)	54.00 c.c.
Tetrachlorethane (tech.)[1]	40.00 c.c.
Glacial acetic acid	6.00 c.c.

Add the alcohol to the tetrachlorethane in a flask and bring to a temperature not to exceed 70° C. (If it is desired to use methyl alcohol the temperature should not be raised to more than 55° to 60° C.) Add the warm mixture to the powdered methylene blue. Shake vigorously until the dye is completely dissolved; then add slowly the glacial acetic acid to the cold solution. Agitate the flask during addition of acid. Filter the entire volume through a 15 centimeter filter paper. Keep in tightly stoppered bottle. Allow the stain to act for thirty seconds. Steps 5 and 6 of the above may be omitted as the fat is removed by the stain.

Fig. 311.—Ocular Micrometer Disk of Breed and Brew

10. Adjust the microscope so the field of vision is 0.205 millimeter in diameter. This can be done by using a stage micrometer with a 1.9 millimeter (oil-immersion) objective and a 6.4 × ocular; adjust the tube until the field has the required size.

Count the number of bacteria seen in 30 fields. Each field represents one three hundred thousandth part of a c.c. of the milk.

$$\frac{\text{number of bacteria}}{30} \times 300{,}000 = \text{number of bacteria per c.c.}$$

or
number of bacteria × 10,000

11. For some purposes. especially when examining low count milk. it is advisable to use a special ocular micrometer with circular ruling divided into quadrants (Fig. 311). Adjust the microscope so the diameter of the circle is 0.146 millimeter. Count 60 fields and multiply the number of bacteria by 10,000.

[1] Obtained from Eastman Kodak Co.

12. This direct microscopic method is excellent for making a rapid survey of either raw or pasteurized milk and should be more frequently used as a check on counts obtained by the plate method.

METHYLENE BLUE REDUCTION METHOD

This test is also known as the reductase test. It is based on the principle that the color imparted to milk by a small quantity of methylene blue will disappear more or less quickly. The rate of this decolorization depends largely upon the reducing activity of bacteria. This in turn has been empirically correlated with the number of bacteria when the test is conducted under the usual circumstances and if other factors such as the temperature of the milk, are kept constant during the test.

1. Collect samples with same care as for other types of bacteriological examinations.

2. Place 10 c.c. of the milk in a sterile test tube.

3. Add 1 c.c. of methylene blue solution made by adding 1 tablet to 200 c.c. of water (standard methylene blue tablets are prepared by the National Aniline Company and can be obtained from the usual supply houses).

4. Mix the dye thoroughly. This can be done by blowing through the pipet used to add the dye. The milk should now have a robin's-egg blue color.

5. Place the tubes in a water bath at 37° C.

6. Observe frequently. The end-point is to be taken as the time when the blue color has disappeared and the milk has regained its normal color. In the majority of cases the color disappears uniformly throughout the entire mass of milk. With certain samples the color may persist at the surface, or again it may persist at the bottom of the tube. In case the color disappears in an uneven manner, the end-point can be taken as the time when the milk after mixing shows no evidence of a blue color.

7. The results are interpreted as follows:

Class 1. Good milk: not decolorized in 5½ hours; developing as a rule, less than 500,000 colonies per c.c. on agar plates.

Class 2. Milk of fair quality: decolorized in less than 5½ hours but not less than 2 hours; developing as a rule, 500,000 to 4,000,000 colonies per c.c. on agar plates.

Class 3. Unsatisfactory milk: decolorized in less than 2 hours, but not less than 20 minutes; developing as a rule, 4,000,000 to 20,000,000 colonies per c.c. on agar plates.

Class 4. Very unsatisfactory milk: decolorized in 20 minutes or less; developing as a rule, over 20,000,000 colonies per c.c. on agar plates.

METHODS FOR THE RECOGNITION OF HEMOLYTIC STREPTOCOCCI

The following methods are useful for the examination of samples from cows suspected of having caused an outbreak of septic sore throat. They may also be used for routine control in those cases where raw milk is carefully guarded against infection with hemolytic streptococci.

1. Beef infusion agar is melted, cooled to 50° C. and 0.5 c.c. of defibrinated blood added for each 10 c.c. of agar.

2. Add 1.0 c.c. and 0.1 c.c. of milk respectively to 2 Petri dishes, overpour with agar and mix thoroughly by gently rotating the plate.

3. Incubate 24 hours at 37° C. Use an uninoculated plate as a sterile control. Plates showing no growth are incubated for an additional 24 hours.

4. Three types of streptococci may be encountered. The first group, the lactic acid streptococci, are not generally active on blood agar. The second type is that of bovine mastitis which appears as a viridans (green), or weakly hemolytic colony. The third group is the highly hemolytic and contains the human type, of which *Streptococcus epidemicus* is a member. This organism is usually associated with septic sore throat epidemics.

5. Pick one or two hemolytic colonies to (*a*) glucose serum broth and to (*b*) sodium hippurate broth. *Glucose serum broth* is prepared by adding 1.0% glucose to beef infusion broth. Two drops of serum are added at the time of inoculation. *Sodium hippurate broth* is prepared by adding 1.0% sodium hippurate to pork or beef infusion broth.

6. After 2 days at 37° C. the final pH of the glucose serum broth culture is determined colorimetrically.

7. At the same time the sodium hippurate culture is examined by adding 1 part of the test reagent (12% ferric chloride in a 2.0% HCl solution) to 4 parts of the culture. Mix and observe after 10 minutes. A precipitate (ferric benzoate) indicates that the hippuric acid has been hydrolyzed.

8. If the suspicious organism produces a final pH of from 6.0 to 5.0 and fails to hydrolyze the hippurate, a moist Indian ink preparation should be made from the serum broth. Should an encapsulated organism be found, it is probably *Strept. epidemicus*. This organism usually ferments salicin but not mannitol.

METHOD FOR THE DETECTION OF TYPHOID AND PARATYPHOID BACILLI

1. Leifson recommends culturing 9 parts of milk in 1 part of selenite enrichment medium prepared in a concentration 10 times stronger than usual. If the milk is of poor quality it is advised to dilute at 1:4 with sterile water and add 9 parts of this dilution to 1 part of the concentrated medium.

2. Incubate at 37° C. for 18 to 24 hours and then prepare surface streak plates of desoxycholate-citrate agar or bismuth sulphite agar. If suspicious colonies are found, apply further tests for typhoid and paratyphoid bacilli as described in Chapter XIX.

CHAPTER XXIV

METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF WATER

Principles.—1. The methods here given are the standard methods for the examination of water adopted by the American Public Health and American Water Works Associations.

2. From the standpoint of the clinical laboratory, examinations for bacilli of the coli-aerogenes group of fecal origin are of most value in relation to the spread of typhoid fever, cholera, and dysentery.

3. Direct examinations for typhoid and paratyphoid bacilli have been previously referred to but are of so very little value that most reliance is placed upon finding colon bacilli as indicative of possible contamination with human fecal material.

COLLECTION

1. Samples for bacterial analysis shall be collected in bottles which have been cleansed with care, rinsed in clean water, and sterilized.

2. Great care must be exercised to have the samples representative of the water to be tested and to see that no contamination occurs at the time of filling the bottles or prior to examination.

3. Because of the rapid and often extensive changes which may take place in the bacterial flora of bottled samples when stored even at temperatures as low as 10° C., it is urged, as of importance, that all samples be examined as promptly as possible after collection.

4. The time allowed for storage or transportation of a bacterial sample between the filling of the sample bottle and the beginning of the analysis should be *not more than 6 hours for impure waters and not more than 12 hours for relatively pure waters.* During the period of storage, the temperature shall be kept between 6° C. and 10° C. Any deviation from the above limits shall be so stated in making reports.

TOTAL BACTERIAL COUNTS

1. Have ready the following: (*a*) Nutrient extract agar or nutrient gelatin with pH of 6.4 liquefied and cooled to 42° C. (*b*) Dilution bottles containing 9 c.c. or 99 c.c. of water and sterilized at 120° C. for 15 minutes.

2. When dilutions are made the sample bottle should be shaken vigorously 25 times and 1 c.c. withdrawn and added to the proper dilution bottle as required. Each dilution bottle shall be shaken vigorously 25 times before a second dilution is made from it or before a sample is removed for plating.

3. Plating should be done immediately after making dilutions. After vigorous

shaking 25 times, 1 c.c. of the sample or dilution shall be placed in the Petri dish. Ten c.c. of liquefied medium (agar or gelatin) at a temperature of 42° C. shall be added to the Petri dish. The cover of the Petri dish shall be lifted just enough for the introduction of the pipet or culture medium, and the lips of all test tubes or flasks used for pouring the medium shall be flamed. The medium and sample in the Petri dish shall be thoroughly mixed and uniformly spread over the bottom of the Petri dish by tilting and rotating the dish. All plates shall be solidified as rapidly as possible after pouring and placed immediately in the incubator.

4. *Gelatin plates* shall be incubated for 48 hours at 20° C. in a dark, well-ventilated incubator in an atmosphere practically saturated with moisture.

Agar plates may be used for counts made either at 20° C. or 37° C. The time for incubation at 20° C. shall be 48 hours and at 37° C. 24 hours. The incubator shall be dark, well-ventilated and the atmosphere shall be practically saturated with moisture. Glass-covered plates shall be inverted in the incubator. Any deviation from the above described method shall be stated in making reports.

In making report of the water examination the medium used for the total count should be stated, *i.e.*, whether gelatin or agar, and the temperature of incubation given.

5. In preparing plates, such amounts of the water under examination shall be planted as will give from 30 to 300 colonies on a plate; and the aim should be to always have at least 2 plates giving colonies between these limits. Where it is possible to obtain plates showing colonies within these limits, only such plates should be considered in recording results, except where the same amount of water has been planted in 2 or more plates, of which one gives colonies within these limits, while the others give less than 30 or more than 300. In such case, the result recorded should be the average of all the plates planted with this amount of water. Ordinarily it is not desirable to plant more than 1 c.c. of water in a plate; therefore, when the total number of colonies developing from 1 c.c. is less than 30, it is obviously necessary to record the results as observed, disregarding the general rule given above.

Counting shall in all cases be done with a lens of 2.5 diameters' magnification, with a focal distance of 3½ inches. The Engraver's Lens No. 146 made by the Bausch and Lomb Optical Company fills the requirements and is a convenient lens for the purpose.

6. In order to avoid fictitious accuracy and yet to express the numerical results by a method consistent with the precision of the work, the numbers of colonies of bacteria per c.c. shall be recorded as follows:

From 1 to 50 shall be recorded as found.
From 51 to 100 shall be recorded to the nearest 5.
From 101 to 250 shall be recorded to the nearest 10.
From 251 to 500 shall be recorded to the nearest 25.
From 501 to 1000 shall be recorded to the nearest 50.

From 1001 to 10,000 shall be recorded to the nearest 100.

From 10,001 to 50,000 shall be recorded to the nearest 500.

From 50,001 to 100,000 shall be recorded to the nearest 1000.

From 100,001 to 500,000 shall be recorded to the nearest 10,000.

From 500,001 to 1,000,000 shall be recorded to the nearest 50,000.

From 1,000,001 to 10,000,000 shall be recorded to the nearest 100,000.

This applies to the gelatin count at 20° C. and to the agar counts at 20° C. and 37° C.

DETERMINATION OF THE PRESENCE OF MEMBERS OF THE COLI-AEROGENES GROUP

The coli-aerogenes group is to be considered as including all gram-negative non-spore-forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media.

The test described under this heading is really a combination of three tests. The first is called the "presumptive test" and is conducted in all cases. The second is called the "confirmed test" and is used to confirm the first test when doubtful. The third is the "completed test," used when the results of the second test are doubtful.

Presumptive Test.—1. Inoculate a series of fermentation tubes containing lactose broth (nutrient broth containing 0.5% lactose) with the following amounts of the water to be tested: 10, 1, 0.1, and 0.01 c.c.

2. The amount of media should always equal at least twice the amount of water inoculated. Any type of fermentation tube can be used. The Durham tube with inverted vial is recommended. When required to examine larger amounts than 10 c.c., as many tubes as necessary shall be inoculated with 10 c.c. each.

3. Incubate at 37° C. for 48 hours.

4. Examine each tube at the end of 24 and 48 hours.

5. The production within 24 hours of gas occupying more than 10% of the inverted vial in the fermentation tube constitutes a *positive presumptive test.*

6. If no gas is formed in 24 hours, or if the gas formed is less than 10%, the incubation shall be continued to 48 hours. The presence of gas in any amount in such a tube at 48 hours constitutes a *doubtful test*, which in all cases requires confirmation.

7. The absence of gas formation after 48 hours' incubation constitutes a *negative test.*

Confirmed Test.—1. Streak or spread Endo or eosin-methylene blue plates from the tube which shows gas formation from the smallest amount of water tested. The transfer should be made as soon as possible after gas formation occurs. If gas formation occurs at the end of 24 hours, make transfer at that time. If at the end of 48 hours gas has formed in tubes containing less of the sample of water than at 24 hours, transfers should be made from these tubes.

2. Incubate at 37° C. for 18 to 24 hours.

3. The results are interpreted as follows: (a) If typical colonies have developed upon the plate within this period, the confirmed test may be considered positive. (b) If, however, no typical colonies have developed within 24 hours, the test cannot yet be considered definitely negative, since it not infrequently happens that members of the coli-aerogenes group fail to form typical colonies on Endo or eosin-methylene blue plates, or that the colonies develop slowly. In such case, it is always necessary to complete the test as described below.

Completed Test.—1. (a) *From typical plates*: From the Endo or eosin-methylene blue plates showing typical colonies, fish at least two colonies, transferring each to an agar slant and a lactose fermentation tube.

(b) *From atypical plates:* If no typical colonies appear upon the plate within 24 hours, the plate should be incubated another 24 hours, after which at least 2 of the colonies considered most likely to be organisms of the coli-aerogenes group whether typical or not shall be transferred to agar slants and lactose fermentation tubes.

2. Incubate the lactose broth fermentation tubes until gas formation is noted, the incubation not to exceed 48 hours. The agar slants shall be incubated 37° C. for 24 hours, when a microscopic examination shall be made of at least one culture, selecting the one which corresponds to one of the lactose broth fermentation tubes which has shown gas formation.

3. The formation of gas in lactose broth and the demonstration of gram-negative non-spore-forming bacilli in the agar culture shall be considered a satisfactorily completed test, demonstrating the presence of a member of the coli-aerogenous group. The absence of gas formation in lactose broth or failure to demonstrate gram-negative non-spore-forming bacilli in a gas-forming culture constitutes a negative test.*

Interpretation of the Results.—*Presumptive Test.*—1. When definitely positive, that is, showing more than 10% of gas in 24 hours, this test is sufficient:

(a) As applied to all except the smallest gas-forming portion of each sample in all examinations.

(b) As applied to the smallest gas-forming portion in the examination of sewage or of water showing relatively high pollution, such that its fitness for use as drinking water does not come into consideration. This applies to the routine examination of raw water in connection with control of the operation of purification plants.

2. When definitely negative, that is, showing no gas in 48 hours, this test is final and therefore sufficient in all cases.

3. When doubtful, that is, showing gas less than 10% (or none) in 24 hours, with gas either more or less than 10% in 48 hours, this test must always be confirmed.

Confirmed Test.—1. When definitely positive, that is, showing typical plate colonies within 24 hours, this test is sufficient:

(a) When applied to confirm a doubtful presumptive test in cases where the latter, if definitely positive, would have been sufficient.

(*b*) In the routine examination of water supplies where a sufficient number of prior examinations have established a satisfactory index of the accuracy and significance of this test in terms of the completed test.

2. When doubtful, that is, showing colonies of doubtful or negative appearance in 24 hours, this test must always be completed.

Completed Test.—The completed test is required as applied to the smallest gas-forming portion of each sample in all cases other than those noted as exceptions under the "presumptive" and the "confirmed" tests.

The completed test is required in *all* cases where the result of the partially confirmed test has been doubtful.

Note.—1. In reporting a single test, it is preferable merely to record results as observed, indicating the amounts tested and the result in each, rather than to attempt expression of the result in number of organisms per c.c. In summarizing the results of a series of tests, however, it is desirable, for the sake of simplicity, to express the results in terms of the number of coli-aerogenes organisms per c.c., or per 100 c.c. The number per c.c. is the reciprocal of the smallest portion (expressed in c.c.) giving a positive result. For example, the result 1 c.c. plus, 0.1 c.c. plus, 0.01 c.c. negative, would be recorded as 10 per c.c. An exception should be made in the case where a negative result is obtained in an amount larger than the smallest portion giving a positive result; for example, in a result such as 10 c.c. plus, 1 c.c. minus, 0.1 c.c. In such case, the result should be recorded as indicating a number of coli-aerogenes organisms per c.c. equal to the reciprocal of the portion next larger than the smallest one giving a positive test, this being a more probable result.

Where tests are made in amounts larger than 1 c.c. giving average results less than 1 per c.c., it is more convenient to express results per one hundred cubic centimeters.

2. Recent work seems to indicate that the coli-aerogenes group as herein defined consists of organisms of both fecal and nonfecal origin. Methods for making this distinction are the methyl red, Voges-Proskauer, indol, and sodium citrate tests, but none have been as yet adopted as standard. This statement should not be construed as detracting from the value of the group test as above described for the routine examination of water supplies.

DIFFERENTIATION OF THE COLI-AEROGENES GROUP INTO FECAL AND NONFECAL TYPES

The coli-aerogenes group contains organisms of both fecal and nonfecal origin. For a satisfactory differentiation the following tests are required:

1. *Indol.*

2. *Voges-Proskauer.*

3. *Methyl Red;* inoculate a 5 c.c. portion of methyl red Voges-Proskauer broth. Incubate 24 hours at 37° C. Add 5 drops of methyl red indicator solution. A distinct red color is positive; a yellow color is negative.

4. *Sodium citrate:* inoculate Koser's citrate broth with a loopful of culture or with a needle. Incubate for 3 days at 37° C. *B. coli* fails to grow in this medium.

REACTIONS OF THE COLI-AEROGENES GROUP

	Indol	Methyl Red	Voges-Proskauer	Citrate
Fecal	+	+	−	−
Nonfecal	−	−	+	+

Intermediate types are common and difficult to interpret.

CHAPTER XXV

METHODS FOR TESTING DISINFECTANTS

A very large number of methods have been proposed for testing the bactericidal and bacteriostatic (antiseptic) properties of disinfectants and the method of testing has a tremendous influence upon the results. For this reason one laboratory may report a substance as possessing a high disinfectant value and another that it is practically inert. It is easily possible, therefore, to influence greatly the value placed upon a disinfectant by the method of testing. It is hoped that the methods here given will prove serviceable for the purposes of the clinical laboratory.

The standardization of disinfectants and antiseptics is based upon their disinfecting power in comparison with phenol. The ratio is expressed as the *phenol coefficient*, which is a figure expressing the ratio of the germicidal efficiency of a disinfectant compared with phenol tested under identical conditions. It is not based on a comparison of different time intervals but on a comparison of different concentrations acting for specified periods of time at designated temperatures.

At present there are in general use three methods of determining the phenol coefficient: The U. S. Hygienic Laboratory Method (Reprint No. 675, U. S. Pub. Health, Reg., 1921, 36, 1559), the Rideal-Walker Method, and the Method of the Food and Drug Administration (F. D. A. Method) given below.

The latter designed by Shippen and Reddish (Circular No. 198, U. S. Dept. of Agriculture, December, 1931) is based upon the Hygienic Laboratory and Rideal-Walker Methods and is particularly valuable in the curtailment of labor, time and material as well as being adapted to the use of *Staphylococcus aureus* and other organisms as well as for *B. typhosus* (*E. typhi*).

FOOD AND DRUG ADMINISTRATION METHOD *

Test Organism and Culture Medium.—1. The test organism is a 22-26 hour culture of *Eberthella typhi* (Hopkins strain) incubated and grown in nutrient broth at 37° C. The broth contains the following ingredients: 5 gm. of Liebig's beef extract, 5 gm. of chemically pure sodium chloride, and 10 gm. of Armour's Peptone (for disinfectant testing) in 1,000 c.c. of distilled water. The mixture is boiled for 20 minutes, made up to original weight (or volume) with distilled water, and adjusted with NaOH to pH 6.8 using the colorimetric method. It is then filtered through paper, tubed (10 c.c. to each tube) and the tubes plugged with cotton and sterilized at 15 pounds pressure for 40 minutes.

2. The test culture is transferred daily in this medium for not more than one month. At the end of each month, a fresh transfer is made from the stock culture. The stock culture is carried on agar slants of the same composition as the broth

* U. S. Dept. Agriculture, Circular No. 198, Dec., 1931.

medium plus 1½% Bacto-Agar (Difco) adjusted to pH 7.2 to 7.4. This medium
is also filtered, tubed, plugged with cotton, sterilized and slanted. The stock culture
is transferred once a month, and the test organism is taken from the month old
stock culture. When the test organism has not been transferred daily, it is advisable
to make 4 or 5 consecutive daily transfers in broth before using it for testing pur-
poses, to be reasonably sure of its conforming to the phenol resistance requirements.
When only one transfer has been skipped, the following transfer from the 48-hour
culture is usually satisfactory for use after 24 hours. Transfers are made with the
platinum loop used in the test. Only cultures giving readings within the following
limits are considered satisfactory:

TABLE I

	5 Minutes	10 Minutes	15 Minutes
Phenol			
1-90	+	+	0
1-100	+	+	+
or			
1-90	0	0	0
1-100	+	+	0

TABLE II

	5 Minutes	10 Minutes	15 Minutes
Phenol			
1-90	+	0	0
1-100	+	+	+

The reading in Table II is that most usually obtained and is the most convenient.

Phenol.—The phenol used must meet the requirements of the U.S.P. and in
addition the congealing point must not be below 40° C. A 5% solution may
be used as a stock solution if kept in a relatively cool place in well stoppered
amber-colored bottles protected from the light. This 5% solution should be
standardized with decinormal bromine or with sodium bromide and bromate
solution.

Apparatus.—1. Besides a number of accurately graduated pipets, 100 c.c.
glass-stoppered graduates or volumetric flasks are almost essential for the making
of correct dilutions. All pipets and graduates should be standardized. The test tubes
for containing the dilutions should be large enough to permit transfers being made
without touching the sides with the transfer needle. Lipped pyrex (to withstand
constant flaming) test tubes 25 by 150 mm. serve very well as these seeding or
medication tubes.

2. A water bath for holding the dilutions at the desired temperature must be
provided. To maintain the temperature practically constant during the period of
the test, the bath should be made so as to contain a relatively large volume per

surface area, and should be insulated. The lid is made with well-spaced holes admitting the 25 mm. tube, but not the lip.

3. The most convenient form of subculture tubes (tubes containing medium for incubating the tested organisms, as well as for growing the test culture) are ordinary nonlipped bacteriological test tubes 20 by 150 mm.

4. The racks for holding the subculture tubes may be of any convenient style. Blocks of wood with a series of holes bored in them are quite satisfactory. Dimensions depend somewhat on the side of the incubator, but the holes should be well spaced to insure quick selection and easy manipulation during the test. It is an added convenience to have the holes large enough to admit the medication tubes while dilutions are being made.

5. The transfers are made with 4 mm. (inside diameter) single loop of number 23B and S. gage platinum wire 1½ to 3 inches long, set in a suitable holder such as an aluminum or glass rod approximately 0.5 cm. in diameter.

Procedure.—1. One per cent stock dilutions of the substance to be tested (or any other convenient dilution of the disinfectant, depending on the strength) are made up, usually in glass-stoppered cylinders or volumetric flasks from which the individual dilutions are then prepared. For rapid routine work the final dilutions may be made directly in the medication tubes. In this case all excess over 5 c.c. must be removed. For more precise work, and when high dilutions are required or volatile substances are dealt with, it is preferable to make up all of the dilutions in volumetric flasks, and then transfer 5 c.c. of the final dilution to the medication tubes. The accompanying tables taken from the Hygienic Laboratory Method show how varying dilutions may be prepared from stock 5% (1:20) and 1% (1:100) solutions of the disinfectant being tested.

2. These tubes containing 5 c.c. of each dilution (including the phenol control) are placed in the water bath at 20° C. for 5 minutes until the temperature of the bath is reached. Even slight variations in temperature may affect the results. The dilutions should cover the range of the killing limits of the disinfectants within 5 and 15 minute periods, and should at the same time be spaced sufficiently close together to insure the desired accuracy.

3. Five-tenths of a cubic centimeter of the test culture is then added to each of the dilutions at the time interval corresponding to the interval at which the transfers are to be made. Thus by the time 10 tubes have been seeded at 30-second intervals, 4½ minutes will have elapsed and a 30-second interval intervenes before the transference to the subculture is commenced. The culture is added from a graduated pipet holding sufficient culture to seed all the tubes in any one set. The pipet may be loosely plugged with cotton at the mouth end before sterilizing, as a precautionary measure. Unfiltered culture is used but it should be thoroughly shaken 15 minutes before use, and allowed to settle. The temperature of the culture should be practically that of the water bath before being added.

4. In inoculating the medication tubes they should be held in a slanting position, after removal from the bath, and the culture run in without the tip of the

pipet touching the disinfectant. The tip may be allowed to rest against the side of the tube just above the surface of the liquid.

5. The tubes are agitated gently but thoroughly after the addition of the culture to insure even distribution of the bacteria.

5 c.c. of disinfectant + 95 c.c. of distilled water = Solution A

		Solution A, c.c.		Distilled Water, c.c.		Solution A, c.c.		Distilled Water, c.c.		Solution A, c.c.		Distilled Water, c.c.
1: 20	=	20	+	0	or	10	+	0	or	4	+	0
1: 25	=	20	+	5	or	10	+	2.5	or	4	+	1
1: 30	=	20	+	10	or	10	+	5	or	4	+	2
1: 35	=	20	+	15	or	10	+	7.5	or	4	+	3
1: 40	=	20	+	20	or	10	+	10	or	4	+	4
1: 45	=	20	+	25	or	10	+	12 5	or	4	+	5
1: 50	=	20	+	30	or	10	+	15	or	4	+	6
1: 55	=	20	+	35	or	10	+	17.5	or	4	+	7
1: 60	=	20	+	40	or	10	+	20	or	4	+	8
1: 65	=	20	+	45	or	10	+	22.5	or	4	+	9
1: 70	=	20	+	50	or	10	+	25	or	4	+	10
1: 80	=	20	+	60	or	10	+	30	or	4	+	12
1: 90	=	20	+	70	or	10	+	35	or	4	+	14
1:100	=	20	+	80	or	10	+	40	or	4	+	16
1:110	=	20	+	90	or	10	+	45	or	4	+	18
1:120	=	20	+	100	or	10	+	50	or	4	+	20
1:130	=	20	+	110	or	10	+	55	or	4	+	22
1:140	=	20	+	120	or	10	+	60	or	4	+	24
1:150	=	20	+	130	or	10	+	65	or	4	+	26
1:160	=	20	+	140	or	10	+	70	or	4	+	28
1:170	=	20	+	150	or	10	+	75	or	4	+	30
1:180	=	20	+	160	or	10	+	80	or	4	+	32
1:200	=	20	+	180	or	10	+	90	or	4	+	36
1:225	=	20	+	205	or	4	+	41	or	2	+	20 5
1:250	=	20	+	230	or	4	+	46	or	2	+	23
1:275	=	20	+	255	or	4	+	51	or	2	+	25.5
1:300	=	20	+	280	or	4	+	56	or	2	+	28
1:325	=	20	+	305	or	4	+	61	or	2	+	30 5
1:350	=	20	+	330	or	4	+	66	or	2	+	30
1:375	=	20	+	355	or	4	+	71	or	2	+	33.5
1:400	=	20	+	380	or	4	+	76	or	2	+	38
1:450	=	20	+	430	or	4	+	86	or	2	+	43
1:500	=	20	+	480	or	4	+	96	or	2	+	48

6. Five minutes from the time of seeding the first medication tube, transfer 1 loopful of the mixture of culture and diluted disinfectant from the medication tube to the corresponding subculture tube. To facilitate transfer of uniform drops of the medication mixture, the loop is bent to form a slight angle with the stem and the medication tube is held at an angle of 60 degrees. In other words, as the loop is withdrawn, its plane should be parallel with the surface of the liquid. At the end of 30 seconds, a loopful is transferred from the second medication tube to the second subculture tube and the process continued for each successive dilution. Five minutes from the time of making the first transfer, a second set of transfers is begun for the 10-minute period, and finally repeated for the 15-minute period.

7. Before each transfer the loop is heated to red heat in the Bunsen flame and

the mouth of every tube is flamed. Sterilization of the loop is effected immediately after making the previous transfer (before replugging the tubes) to allow time for sufficient cooling. Time does not permit flaming the tubes after making the transfer. For this reason, care in transferring and seeding is necessary. Due caution is observed to prevent either the seeding pipet or the transfer needle from touching the sides or mouth of these.

1 c.c. of disinfectant + 99 c.c. of distilled water = Solution B

Dilution		Solution B, c.c.		Distilled Water, c.c.		Solution B, c.c.		Distilled Water, c.c.		Solution B, c.c.		Distilled Water, c.c.
1:100	=	100	+	0	or	10	+	0				
1:110	=	100	+	10	or	10	+	1				
1:120	=	100	+	20	or	10	+	2				
1:130	=	100	+	30	or	10	+	3				
1:140	=	100	+	40	or	10	+	4				
1:150	=	100	+	50	or	10	+	5				
1:160	=	100	+	60	or	10	+	6				
1:180	=	100	+	80	or	10	+	8				
1:200	=	100	+	100	or	10	+	10	or	4	+	4
1:225	=	100	+	125	or	10	+	12.5	or	4	+	5
1:250	=	100	+	150	or	10	+	15	or	4	+	6
1:275	=	100	+	175	or	10	+	17.5	or	4	+	7
1:300	=	100	+	200	or	10	+	20	or	4	+	8
1:325	=	100	+	225	or	10	+	22.5	or	4	+	9
1:350	=	100	+	250	or	10	+	25	or	4	+	10
1:375	=	100	+	275	or	10	+	27.5	or	4	+	11
1:400	=	100	+	300	or	10	+	30	or	4	+	12
1:450	=	10	+	35	or	4	+	14	or	2	+	7
1:500	=	10	+	40	or	4	+	16	or	2	+	8
1:550	=	10	+	45	or	4	+	18	or	2	+	9
1:600	=	10	+	50	or	4	+	20	or	2	+	10
1:650	=	10	+	55	or	4	+	22	or	2	+	11
1:700	=	10	+	60	or	4	+	24	or	2	+	12
1:750	=	10	+	65	or	4	+	26	or	2	+	13
1:800	=	10	+	70	or	4	+	28	or	2	+	14
1:850	=	10	+	75	or	4	+	30	or	2	+	15
1:900	=	10	+	80	or	4	+	32	or	2	+	16
1:1000	=	5	+	45	or	4	+	36	or	2	+	18
1:1100	=	5	+	50	or	4	+	40	or	2	+	20
1:1200	=	5	+	55	or	4	+	44	or	2	+	22
1:1300	=	5	+	60	or	4	+	48	or	2	+	24
1:1400	=	5	+	65	or	4	+	52	or	2	+	26
1:1500	=	5	+	70	or	4	+	56	or	2	+	28
1:1600	=	5	+	75	or	4	+	60	or	2	+	30
1:1700	=	5	+	80	or	4	+	64	or	2	+	32
1:1800	=	5	+	85	or	4	+	68	or	2	+	34
1:2000	=	5	+	95	or	4	+	76	or	2	+	38
1:2200	=	5	+	105	or	4	+	84	or	2	+	42
1:2400	=	5	+	115	or	4	+	92	or	2	+	46
1:2600	=	5	+	125	or	4	+	100	or	2	+	50
1:2800	=	5	+	135	or	4	+	108	or	2	+	54
1:3000	=	5	+	145	or	4	+	116	or	2	+	58
1:3200	=	5	+	155	or	4	+	124	or	2	+	62

8. After completion of the transferring, the subculture tubes are incubated at 37° C. for 48 hours and results read. Macroscopic examination suffices for this, but occasionally agglutination with antityphoid serum will aid in reading doubtful re-

ѕ. A 3-day incubation period or agar streak or microscopic examination may
esorted to in determining feeble growth, especially when organisms other than
rthella typhi are used.

here are certain types of germicidal agents, such as many of the mercury
pounds, which give very high results by phenol coefficient tests. Due to the
ι inhibitory value of such substances in preventing growth in the subcultures
e figures are frequently misleading. For germicides used in the disinfection of
ι objects as surgical instruments, this is of particular importance and must
aken into account. Failure to appreciate this characteristic of certain com-
nds is much more likely to lead to error when *Staphylococcus aureus* is used
er than *Eberthella typhi* as the test organism. That false values may not be
ined for products of this type, or for any other disinfectant giving suspiciously
ι results, the subcultures should contain very large amounts of medium (not
than 200 c.c.) or they should be transferred by carrying at least 4 loopsful
n the first subculture to a second tube of broth as recommended by Shippen.

ther groups of disinfectants in common use, for which the phenol coefficient
hod of testing is not well adapted, are those compounds containing chlorine
he active agent as well as oxidizing agents in general. These are affected so
erially by the presence of organic matter that a phenol coefficient statement
ʳ grossly misrepresent their value under practical conditions of use and is
ʳ apt to be misleading to the consumer when placed on the label.

alculation of the Phenol Coefficient.—1. The results of the test are ex-
sed in terms of the phenol coefficient. This represents the germicidal value of
diluted disinfectant as compared with the diluted phenol control. It is a
re obtained by dividing the numerical value of the greatest dilution (the
ominator of the fraction expressing the dilution) of the disinfectant capable
illing *Eberthella typhi* in 10 minutes but not in 5 minutes, by the greatest dilu-
of the phenol showing the same results, that is, by the phenol control. Thus.
ιc results were as follows:

	5 Minutes	10 Minutes	15 Minutes
ΝFΙCΤΑΝΤ (X)			
1-300	0	0	0
1-325	+	0	0
1-350	+	0	0
1-375	+	+	0
1-400	+	+	+
ΝOL			
1-90	+	0	0
1-100	+	+	+

he phenol coefficient would be $\frac{350}{90}$ equals 3.89

. If none of the dilutions shows growth in 5 minutes and killing in 10 minutes,
hypothetical dilution may be estimated in certain cases. This may be done only
n any three consecutive dilutions show the following results: The first—no

growth in 5 minutes; the second, growth in 10 minutes but not in 15 minutes, and the third, growth in 15 minutes. Example:

	5 Minutes	10 Minutes	15 Minutes
DISINFECTANT (X)			
1-300	0	0	0
1-350	+	+	0
1-400	+	+	+
PHENOL			
1-90	0	0	0
1-100	+	+	+

The estimated phenol coefficient would be $\frac{325}{95}$ equals 3.42.

3. To avoid giving an impression of fictitious accuracy, the phenol coefficient is calculated to the nearest 0.1 unless the coefficient is less than 1.0. Thus, in the examples cited above, the phenol coefficients would be reported as 3.9 and 3.4 instead of 3.89 and 3.42.

In the preceding description, *Eberthella typhi* has been mentioned as the test organism. Wherever any expression of phenol coefficient occurs in literature, on labels, etc., it is assumed to mean the *E. typhi* phenol coefficient unless otherwise stated. It is, however, the distinct intention of the U. S. Dept. of Agriculture not to limit the test to the use of one organism. In fact, the test has been found adaptable to the use of a wide variety of bacterial species in the determination of phenol coefficients. In cases where some of the more strictly parasitic bacteria are used, modifications in media are necessitated, and, of course, a change in the phenol dilutions. Therefore, discussion of the exact technic is here omitted, with the exception of that for *Staphylococcus aureus*. When any test organism other than *E. typhi* is used it should be distinctly designated when stating the phenol coefficient.

Tests with Staphylococcus Aureus.—1. *S. aureus* has been found to be an extremely useful organism for testing disinfectants and antiseptics and has been used for this purpose for a number of years. When substituted in the above test the technic remains exactly the same. The phenol dilutions, however, must be changed. The resistance of any strain of *S. aureus* used in this test must come within the following limits: At 20° C. it must survive a 1:60 dilution of phenol for 5 minutes and a 1:70 dilution for 15 minutes. The following is the minimal resistance that would be acceptable:

	5 Minutes	10 Minutes	15 Minutes
PHENOL			
1-60	+	0	0
1-70	+	+	+

2. In the bacteriological examination of disinfectants, the *Eberthella typhi* and the *S. aureus* phenol coefficients give, in general, sufficient information to render

tests with other organisms unnecessary, except in special instances. The commonly accepted criterion that disinfectants for general use be employed at a dilution equivalent to the germicidal efficiency of 5% phenol against *E. typhi* (that is, 20 times the *E. typhi* phenol coefficient) allows a reasonable margin of safety for the destruction of infective agents likely to be the object of general disinfection about premises with the possible exception of *Mycobacterium tuberculosis*. *S. aureus*, due to its ubiquity, resistance and ever ready tendency to cause infection, should always be employed in testing those substances recommended for personal use or as application for wounds. If the disinfectant is recommended for use externally the temperature of test should be 20° C. but where such substances are recommended for use in the body cavities such as for mouth washes, gargles, douches, etc., this test should be conducted at 37° C. In such case the test should be designated as "The F. D. A. method (special) *S. aureus* 37° C." At body temperature the *S. aureus* should show the following resistance to phenol:

	5 Minutes	10 Minutes	15 Minutes
1-80	+	0	0
1-90	+	+	+
or			
1-80	+	0	0
1-90	+	+	0

KOLMER BACTERIOSTATIC METHODS

This test is of extreme simplicity and yields sharply defined results. It determines the highest dilution of a disinfectant capable of restraining the growth of the test organism for a stated period of time and is of particular value for comparing the antiseptic properties of various chemical agents.

Method Employing Nutrient Bouillon.—For tests with staphylococci, *B. anthracis*, *B. typhosus*, *B. coli* and such hardy organisms, plain beef extract broth with a pH of 7.1 may be employed; Kligler has used a medium prepared of 1% Fairchild's peptone, 0.5% dibasic potassium phosphate, 0.5% sodium chloride and 0.1% glucose with a constant pH of 7.1. The reaction of any medium employed is particularly important as the results may be greatly modified by this factor. For streptococci and pneumococci, hormone broth (Huntoon) with 0.1% dextrose and a pH of 7.7 is to be preferred, and for tubercle bacilli, the ordinary 5% glycerin-hormone broth employed in the manufacture of Koch's tuberculin should be used. *It is always advisable to determine beforehand that the organism will grow well in the medium employed before the tests are conducted.*

1. As a general rule, 10 dilutions are advisable and for this purpose 10 sterile test tubes are arranged for each compound, including a set for the bichloride of mercury or phenol controls.

2. In all tubes except No. 1 of each series. place 1 c.c. of sterile distilled water.

3. In tubes 1 and 2 place 1 c.c. of the stock solution of disinfectant which is

ten times higher than the final dilutions desired. Mix No. 2 and transfer 1 c.c. to No. 3 and so on to No. 10 from which discard 1 c.c.

4. In a flask of 99 c.c. of the culture medium, place 1 c.c. of a 24 to 48 hour broth culture of the test organism; with such organisms as streptococci and pneumococci it is well, however, to seed by mixing 5 c.c. of a broth culture with 95 c.c. of the culture medium. Mix well and add 9 c.c. to each tube of the set; the remaining 10 c.c. are placed in a sterile tube as a control on the culture.

5. In tests employing tubercle bacilli, it is better to add 9 c.c. of sterile medium to each tube and then to seed by floating a loopful of bacilli on the surface of each.

6. The final dilutions are now ten times higher in each tube; for example, 1 c.c. of 1 : 1000 stock solution in No. 1 becomes 1 : 10,000 and the final dilution in No. 10 is 1 : 5,120,000.

7. The tubes are incubated and the results recorded daily for 5 days. When the medium remains clear the result is recorded as *minus*; when a visible growth appears, the result is recorded as *plus*. At the end of this period the tubes may be cultured by transferring several loopfuls to slants of a solid medium to determine whether the organisms have been killed or merely restrained. In this manner a bactericidal test is conducted at the same time in which a few organisms have been exposed to the disinfectant for 5 days. The control should be subcultured at the same time to make sure that the organisms are viable.

8. The results are expressed according to the highest bacteriostatic and bactericidal dilutions and also according to the bichloride or phenol coefficients previously described.

9. After obtaining in this manner an approximate idea of the activity of the compound under study, a second series of dilutions is prepared in which the variations from tube to tube are less marked.

Method Employing Serum, Blood, Ascites Fluid or Muscle Extract.— These tests are conducted in exactly the same manner as described above except that to 89 c.c. of a suitable broth medium are added 10 c.c. of sterile serum, blood or ascites fluid; the mixture is then seeded with 1 c.c. of a broth culture of the test organism; with such organisms as the pneumococcus and streptococcus, however, it is generally advisable to use 85 c.c. of both, 10 c.c. of serum, blood or ascites fluid with 5 c.c. of broth culture of the test organism. This gives a 10% solution of serum, blood, or ascites fluid, and while more or less may be employed as desired, yet experience has indicated that the above is satisfactory for eliciting the influence of these substances upon the degree of antibacterial activity of disinfectants.

Numerous experiments have shown that while the bacteriostatic and bactericidal activity of various disinfectants is reduced to a greater extent on a 50% than on a 5% dilution of serum, defibrinated blood, or ascites fluid, yet for all practical purposes a 10% solution is satisfactory for this purpose and in view of the large amounts required, is to be preferred from the standpoint of economy. With muscle extracts, however, it may be advisable to use equal parts with broth (50%) but muscle extract may be prepared so cheaply and quickly, as required, that the

questions of economy and supply are not involved. These preparations are likewise slightly cloudy but not usually to a degree sufficient for interfering with the ease and accuracy of readings. A menstruum containing 10% of a muscle extract prepared of ordinary beef or veal requires a far higher concentration of disinfectants than a menstruum of 10% blood or serum: this is doubtless due to the presence of large numbers of various bacteria in addition to the test organism.

Other special media like hormone-dextrose broth with brain tissue, ascites broth with sterile kidney, etc., may be employed in tests of this kind, and while the results vary according to the constitution of the medium, yet if mercuric chloride or phenol are included in each and every test, the results may be expressed in terms of the coefficients or indices. The coefficients, however, will vary according to the chemical nature of the compound as this is influenced by the constitution of the medium; for example, some compounds of mercury, like mercurophen and metaphen, maintain a higher degree of bacteriostatic activity in a serum, blood, or brain medium than mercuric chloride and thereby yield higher coefficients. In other words, while the bactericidal activity of almost all disinfectants is reduced in the presence of serum, blood, muscle extract, etc., the degree of reduction varies considerably among different compounds.

Method Employing Solid Media; Mycostatic Test.—It is very easy to employ solid culture media in this technic and for such organisms as grow better on solid than fluid medium, as the various yeasts and molds, the former are to be preferred.

In conducting this test, a series of dilutions of the disinfectant in amounts of 1 c.c. in sterile distilled water are prepared in sterile test tubes as previously described. To each tube and a control are now added 9 c.c. of an appropriate agar medium cooled to 42° to 45° C.: the contents are well mixed and allowed to harden in slants. The dilution in each tube is now 10 times higher; or 4 c.c. of medium may be added to each tube, which renders the final dilution in each 5 times higher.

For such organisms as staphylococci, *B. typhosus*, *B. coli*, etc., plain 2% agar (pH 7.1) may be employed; for streptococci and pneumococci a hormone-dextrose (0.1%) agar with a pH of 7.7 is to be preferred. For tubercle bacilli, a glycerin agar may be employed since an egg medium may be unsatisfactory because heating for inspissation and sterilization may break up some disinfectants. For parasitic molds like *Trichophyton rosaceum*, *Microsporon audouini* and *Achorion schoenleinii*, Sabouraud's maltose medium titrated to + 1.0 to phenolphthalein, may be employed. Whatever medium is chosen, it must be adopted for cultivating the test organism, should be sterile, liquefied by heating, and added to the tubes containing varying dilutions of the disinfectant while still fluid after being cooled to 40° to 45° C. After hardening has occurred, each tube is inoculated in as uniform manner as possible and the tubes incubated for a period of 5 to 10 days (mycostatic tests for two weeks or longer at room temperature) as decided upon. The results are expressed according to the highest dilution of disinfectant capable of preventing the growth of the test organism. Mercuric chloride or phenol controls may be included and the results expressed in terms of coefficients.

METHODS FOR THE COLLECTION OF BLOOD AND SERUM

COLLECTION OF BLOOD

Precautions.—1. Collection from a vein is advisable and the left arm is preferred.

2. Blood should not be taken immediately after a meal but any time after one hour is satisfactory. *It is not necessary for the patient to be fasting.*

3. As far as possible avoid drawing blood for the Wassermann test during febrile periods and acute alcoholism.

4. Sterile containers are advisable, although if the test is to be done within three days the presence of a few organisms is without effect.

5. Five to 10 c.c. of blood are sufficient. *When mailed a small container should be used in order that the contents shall reach almost to the stopper to avoid undue agitation and hemolysis.* The specimen should be carefully wrapped in cotton or other material to protect against breakage, and mailing cases should be those approved by the postal service.

6. *Specimens for mailing should be shipped at once* in order to avoid undue hemolysis and anticomplementary sera. Under these conditions specimens can be shipped long distances. It is advisable to send them by air mail or special delivery.

7. If cotton stoppers are used, due care must be taken to prevent blood from coming in contact with them.

8. *Each specimen should be labeled at once* to prevent the very regrettable and inexcusable mistakes sometimes made. Labels may come off and should be held on with rubber bands.

From the Veins of Adults.—1. Place a tourniquet (not too tightly in order not to shut off arterial circulation) about the arm above the elbow; request patient to open and close the hand vigorously. Select a vein usually best seen or felt in the flexor surface of the elbow (Fig. 312). A vein in the forearm or back of the hand may be used.

2. Cleanse the skin over a prominent vein with tincture of iodine and alcohol.

3. Remove 5 to 10 c.c. of blood with a sterilized Luer or Record syringe fitted with a No. 20 to 22 needle (Fig. 313). Transfer the blood to a small sterile test tube, vial or other suitable container.

4. The Keidel tube is particularly convenient. A modified Keidel tube made by Harris Comer, Philadelphia, enables one to see a flow of blood and, therefore, that the vein has been entered, before the neck of the vial is broken (Figs. 314 and

315). This tube is particularly convenient. After filling with blood, the ampule should be sealed by bending over the rubber stem with a rubber band to prevent loss if specimen is mailed.

5. Release the tourniquet and then withdraw the needle.

6. As a general rule, the wound requires no further attention unless several punctures have been made, in which case it should be cleansed with alcohol and covered with flexible collodion.

From an Artery.—Blood may be safely taken from a radial, brachial, or femoral artery but this is seldom required. The technic is the same as venipuncture. A No. 20 needle is usually adequate. Upon withdrawal the wound should be compressed firmly and long enough to prevent a hematoma. According to Nicholson a greater percentage of positive blood cultures are secured with arterial blood and especially in bacterial endocarditis.

From a Finger.—1. Small amounts of blood for agglutination, Wassermann, transfusion, blood chemistry (micromethods) and other tests are easily obtained by puncturing a finger.

2. The hand must be warm with good circulation. If cold and clammy, have patient immerse the hand for a few minutes in hot water and rub briskly with a towel.

3. Cleanse tip of finger with alcohol; dry.

4. Puncture across the lines at tip of finger with a blood lancet of the spring release type, a one-half pen-nib, a three-cornered surgical needle, or a cataract knife. Cleanse the instrument with alcohol before use.

5. Massage blood into a small test tube; with a large tube too much is lost on the sides (Fig. 316). Small amounts may be collected in a Wright capsule (Fig. 317).

From Infants.—Blood may be obtained at birth by allowing the umbilical cord to bleed about 5 c.c. into a container.
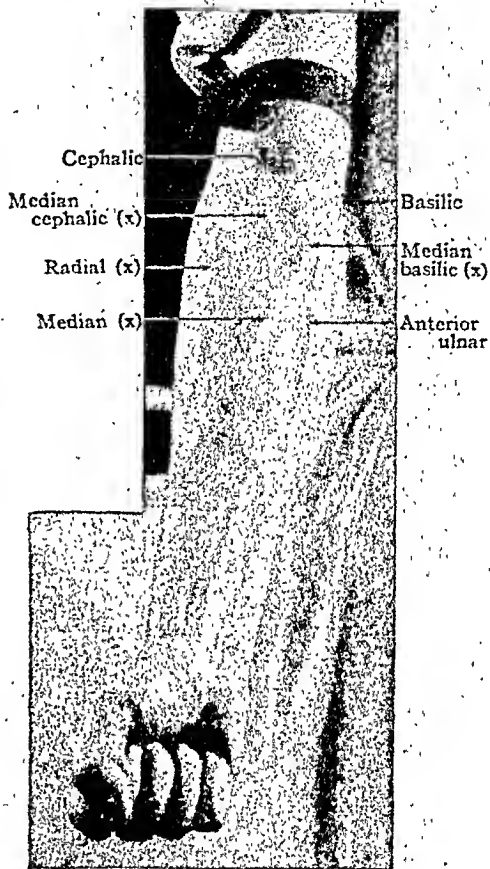
FIG. 312.—CHOICE OF VEINS FOR INTRAVENOUS INJECTIONS AND SECURING BLOOD

The sites of choice are marked with an (x); it is always advisable to avoid punctures near the crease of the elbow in order to avoid subsequent discomfort. (From Kolmer, *Chemotherapy with Special Reference to the Treatment of Syphilis,* W. B. Saunders Co.)

Fig. 313.—Syringe Method for Obtaining Blood
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)

Fig. 314.—Securing Blood with the Keidel Tube; Method of Passing the Needle
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.)
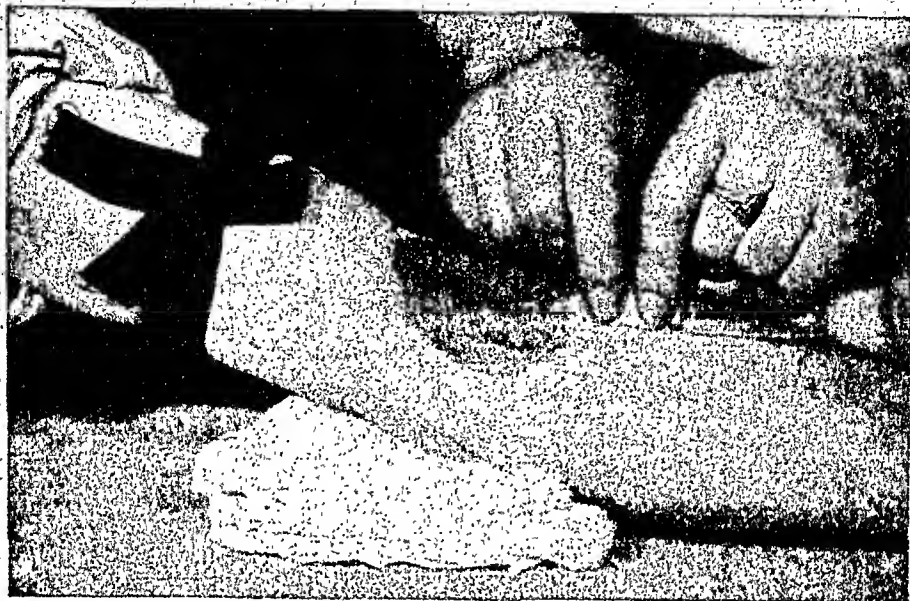
FIG. 315.—SECOND STEP IN SECURING BLOOD WITH THE KEIDEL TUBE.
(From Kolmer, *Chemotherapy*, W. B. Saunders Co.).
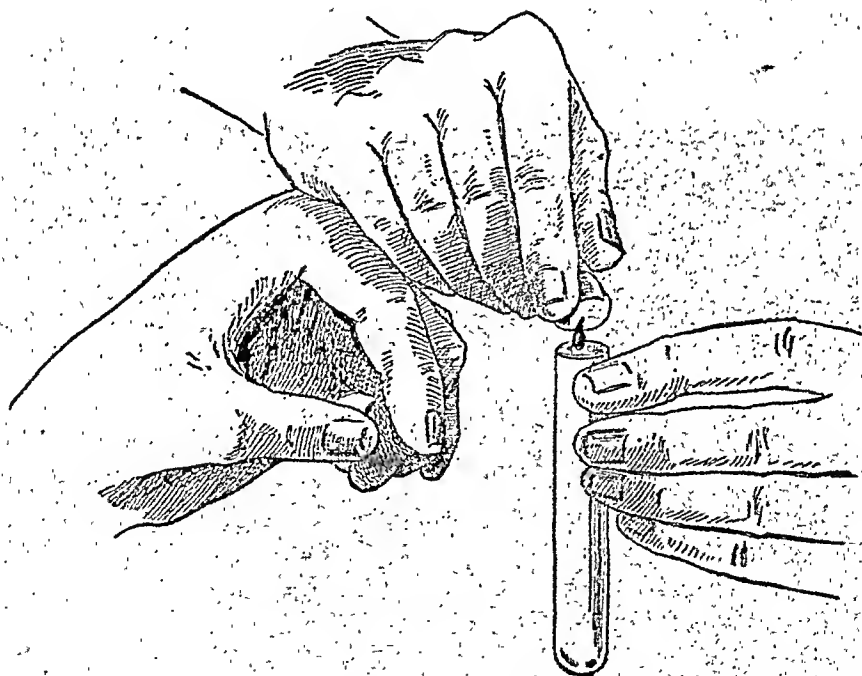
FIG. 316.—SECURING BLOOD FROM A FINGER
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

A few c.c. of blood may be readily obtained from infants by puncturing one of the large toes with a lancet and massaging the blood into a small test tube, as described above for securing blood by puncture of a finger.

In the case of children from 1 to 6 years, sufficient blood may be obtained by puncture of one or several fingers. In children over 4 years of age, a vein may be entered and blood drawn with a Keidel tube or syringe as in the method above described.

Blood may be obtained with a syringe from one of the *external jugular veins*

Fig. 317.—Collecting Blood in a Wright Capsule

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

as follows: (1) Place the infant on its side on a pillow so that the head hangs down on a table or bed. During crying the vein is easily seen and felt. (2) Have an assistant make pressure with an index finger in the supraclavicular fossa to distend the vein. (3) Steady the vein below the angle of the jaw and enter the vein with the needle pointed downward toward the assistant's finger (Fig. 318).

The *long saphenous vein* just anterior to and slightly above the internal malleolus is also serviceable: (1) Wrap the child in a sheet and place at the edge of a bed or table with the buttocks and thighs on a pillow. (2) Apply a tourniquet just below the knee. (3) Have an assistant steady the leg. (4) Prepare the skin and conduct venipuncture with a No. 20 needle attached to a syringe.

Blood may be secured from infants under one year of age by puncture of the *superior longitudinal sinus* as follows:

1. The infant is wrapped in a blanket and the head is steadied by an assistant.

2. The puncture is made on the median line of the posterior angle of the anterior fontanel (Fig. 319).

3. The skin is carefully cleansed. The needle, gage No. 18, with a short bevel, sterilized and attached to a sterile 5 c.c. Record or Luer syringe, is passed inward at a right angle for a distance of about 4 millimeters and suction made; if blood does not flow the needle should be passed about 2 millimeters farther, which suffices for the majority of children up to 15 months of age.

4. At least 3 to 5 c.c. of blood may be safely withdrawn and discharged into a vial. The puncture site is then cleansed and may be sealed with a touch of collodion.

Method for Obtaining Blood at Autopsy.—The heart blood is preferred. After searing the epicardium, a needle or sharp pointed pipet is passed through

FIG. 318.—METHOD OF OBTAINING BLOOD FROM AN EXTERNAL JUGULAR VEIN

The patient is a child six years of age; shows the position of the patient and manner of distending the vein by pressure above the clavicle. A 5 c.c. Record syringe fitted with a No. 20 needle is being used for the withdrawal of blood. The distended vein is painted with tincture of iodin to indicate the position. (From Kolmer in *Keen's Surgery*, W. B. Saunders Co.)
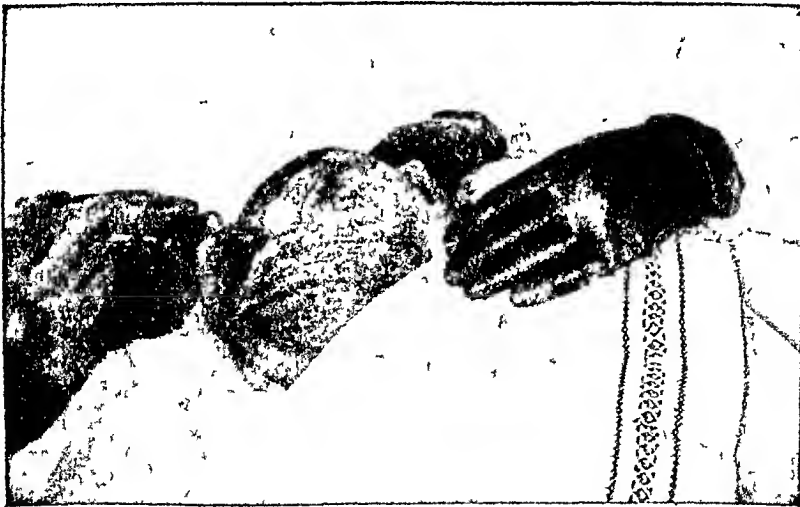
FIG. 319.—METHOD OF OBTAINING BLOOD AND GIVING AN INTRAVENOUS INJECTION BY WAY OF THE SUPERIOR LONGITUDINAL SINUS

The child is six months of age; the shape and size of the anterior fontanel have been outlined; a 5 c.c. syringe fitted with No. 18 needle is being employed, the needle having been entered for about 6 millimeters in the median line at the posterior angle and perpendicular to the sinus. (From Kolmer in *Keen's Surgery*, W. B. Saunders Co.)

the muscle into the right auricle or ventricle. Unless the blood is comparatively fresh, the serum is apt to be anticomplementary.

SEPARATION OF THE SERUM

1. If serum is desired at once, allow the blood to coagulate for a few minutes, break up thoroughly with a rod and centrifuge.
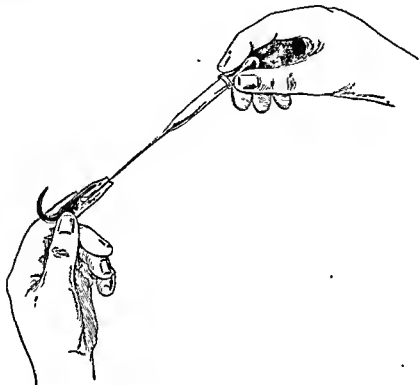
Fig. 320.—Removing Serum from a Wright Capsule

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

2. If serum is not needed at once, place specimen in a refrigerator for spontaneous contraction of the clot and separation of serum (Fig. 320). If this is unsatisfactory, break up the clot with a rod and centrifuge.

METHOD FOR THE COLLECTION AND PREPARATION OF NORMAL AND CONVALESCENT HUMAN SERUM

1. Sterile human convalescent serum is frequently employed for the prophylaxis of scarlet fever, measles, mumps, chickenpox, acute anterior poliomyelitis, etc., and for the treatment of scarlet fever. Normal pooled adult human serum is also employed for the prophylaxis of some diseases as well as for the treatment of hemorrhagic states.

2. Convalescent blood may be taken from donors following recovery or when convalescence is well established.

3. Rigid precautions must be taken against contamination during the collection of blood and preparation of serum because a serum once contaminated should not be used even though subsequently rendered sterile by antiseptics. Severe shock reactions may be produced by residual bacterial protein, denatured serum proteins or bacterial toxins (especially staphylotoxins).

4. Each serum should be examined by the Kolmer complement fixation, Kahn. Kline or Eagle tests and only those giving negative reactions employed for prophylactic and therapeutic purposes.

Method.—1. Adults or older children are preferred for donors. Do not collect blood immediately after a meal in order to avoid chylous sera.

2. Have donor lying down. Carefully cleanse skin over a prominent vein at the elbow and apply tincture of iodine. The method of preparation is the same as for blood transfusion.

3. For the collection of blood use a 500 c.c. Erlenmeyer flask fitted with a two-holed rubber stopper. Into one hole place a short piece of glass tubing connected with rubber tubing for suction by mouth or syringe. Into the second hole place a short piece of glass tubing connected with rubber tubing carrying a gage No. 18 needle. Sterilize in autoclave or by boiling.

4. Apply tourniquet to the arm (not too tightly) ; have patient open and close the hand to distend the veins. Enter the needle into a prominent vein. With suction collect 300 or 400 c.c. of blood. Release the tourniquet; withdraw the needle; remove the iodine with alcohol; apply dressing. Have patient rest 5 minutes and drink a glass of water or milk.

5. Remove the two-holed stopper from the flask and replace with a boiled solid rubber stopper.

6. Allow blood to stand at room temperature for several hours. Rotate the flask to free the clot; place in refrigerator overnight.

7. Carefully pipet the serum to sterile 50 c.c. centrifuge tubes fitted with sterile rubber stoppers or caps and centrifuge thoroughly to remove all corpuscles.

8. Carefully pipet the clear serum to sterile flasks or bottles. Keep the serum of each patient separate.

9. Retain 2 or 3 c.c. for syphilis serological tests and culture 1 or 2 c.c. in 50 c.c. of glucose hormone broth. Incubate at 37° C. for at least 5 to 10 days.

10. Discard all contaminated sera or those giving positive syphilis reactions.

11. Pool the remaining sera in sterile flasks or bottles with rigid aseptic care. To each 100 c.c. add 10 c.c. of 1:500 aqueous solution of metaphen or 1 c.c. of 1:50 aqueous solution of merthiolate. Mix thoroughly.

12. Dispense the serum in sterile vials or bottles with sterile rubber stoppers or caps. It is advisable to culture one or more vials for a final sterility test (incubate 10 days). Both aerobic and anaerobic cultures are desirable.

The finished serum should be crystal clear; free of erythrocytes, bits of fibrin and fats; sterile and Wassermann-negative.

PRESERVATION OF SERUM

1. Sterile serum may be kept in sterile vials or other containers in a refrigerator without a preservative, but this is inadvisable in the case of sera for administration to human beings.

2. Otherwise it is well to add a preservative as 0.5% phenol or tricresol (preferred). Keep a 5% stock solution and add 0.1 c.c. to each c.c. of serum.

3. Rabbit serum hemolysins may be preserved by adding to the serum an equal part of chemically pure glycerin; mix well and keep in refrigerator.

4. Immune sera (antitoxins, agglutinins) may be preserved in dried powder form.

The Lyophile Process for the Preservation of Complement and Human Sera.—An excellent method for preserving normal and immune sera including guinea-pig complement, is by freezing and dehydrating rapidly under high vacuum by an apparatus devised by Flosdorf and Mudd.[1] As described by Mudd, Flosdorf, Eagle, Stokes and McGuinness,[2] the serum or other biologic material to be preserved is distributed with sterile precautions into glass containers. These are immersed in a bath of Dry-ice in a commercial solvent (Methyl Cellosolve) at a temperature of approximately −75° C. The containers of the frozen serum are attached to a manifold which leads through a condenser to a vacuum pump. The condenser is kept at −75° C. with a bath of Dry-ice in the same solvent. The whole system is rapidly evacuated and held at a pressure below 0.05 mm. of mercury by the vacuum pump. Water vapor evaporates from the frozen serum to be trapped as ice in the condenser; the rate of evaporation is sufficient to keep the serum frozen throughout the process of desiccation. The containers are sealed without breaking the original vacuum. Storage for prolonged periods should be at refrigerator temperature. The light porous residue of serum solids quickly and completely dissolves in distilled water to regenerate a serum the potency of which is not detectably different from the original serum. This procedure has been termed the "lyophile" process.

Lyophile serum may be restored to liquid form by the addition of distilled water to one-half or even to one-fourth the original volume. This concentration is of considerable practical advantage, particularly in the routine intramuscular administration of the serum to children, since less painful injections of smaller volumes of liquid are thus made possible.

The blood is collected in 400 c.c. pyrex centrifuge bottles. Fitted on these bottles is a two-holed rubber stopper through which pass 2 glass tubes, one 2 inches and the other 3 inches long. To the 2 inch tube is attached a piece of rubber tubing 18 inches long; this tube contains a cotton plug and to its end is attached a hard rubber mouthpiece for suction. To the 3-inch glass tube is attached an 18-inch rubber tube, to the other end of which is fitted a "Luer-lock" adapter. A 2-inch

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[1] *J. Immunol.*, 1935, 29:389. The apparatus is made by the F. J. Stokes Machine Company of Philadelphia.

[2] *J. A. M. Asso.*, 1936, 107:956.

No. 18 gage needle will give an adequate flow of blood when aided by the reduced pressure caused by the mouth suction and causes the donors little pain.

At the end of the bleeding the suction is stopped, the tourniquet is released about 20 seconds later and the needle is then withdrawn. Enough blood may then be milked from the tube to which the needle is attached for the performance of the Wassermann or Kahn test. After the bleeding, the rubber stopper with the bleeding and suction tubes attached is removed from the bottle. The neck of the bottle is flamed and then closed with a sterile solid rubber stopper. In order to give added protection a piece of sterile lead foil is then placed over the solid stopper and pressed down close to the side of the bottle. A number of bleeding outfits, *i.e.*, the perforated stopper with rubber tubing attached, and centrifuge bottles, are kept on hand and are put up in autoclaved packets.

The blood after collection in centrifuge bottles is stored overnight in the refrigerator. The clot is then "rimmed," the bottle centrifuged and the serum removed with sterile precautions. This is best done by aspiration with negative pressure in a dust-free room. The serum so obtained containing erythrocytes is recentrifugated. and aspiration is repeated without disturbing the red cell sediment. The serum is not mixed with serum from other donors for pooling until a negative Kolmer-Wassermann or Kahn report has been received and until a sterility test has shown the absence of gross contamination of pathogenic bacteria.

To prepare lyophile human serum for intravenous use the foregoing routine is modified. The pooled serum in large volumes is processed without filtration and is then regenerated. The turbid regenerated serum is clarified by passage under 10 atmospheres of air pressure through a Berkefeld V filter (such a filter testing at about 6 pounds). This clarified serum is then refiltered to insure sterility through a Berkefeld W (14 pounds) candle and is distributed into final containers and again lyophile processed. The volume of serum filtered should be as large as practicable in proportion to the size of the filters, in order to minimize losses by adsorption and occlusion in the filters. This doubly filtered, doubly processed serum redissolves to a clear or opalescent solution in as little as one-fourth its original volume of distilled water, and on intravenous administration has the desired clinical effect of reduction of cerebrospinal fluid pressure and increase of arterial blood pressure as well as of immunological benefit.

The Cryochem Process for the Preservation of Complement and Human Sera.—More recently, Flosdorf and Mudd have developed a process for accomplishing the drying of labile biological substances from the frozen state which is considerably cheaper and simpler than the lyophile process. Dry-ice is not required either for initial freezing or for condensation of the evaporated water. A chemical, known as Drierite (anhydrous calcium sulfate, specially prepared at about 200° C.) is used for absorption of the water vapor and is repeatedly regenerable.

The containers of material are attached to the apparatus and the serum pumped essentially free of gases under low vacuum for about a half hour. A high vacuum is then established and the initial freezing is spontaneous, taking place immediately

as a result of the particularly rapid dehydrating action of the Drierite. During the desiccation, not as high a vacuum is required as in the lyophile process and completion of the drying is accomplished in a shorter time.

In many cases the final product has superior solubility and other characteristics, but presents a somewhat less attractive appearance. In cases where appearance might be a major factor, the serum may be frozen initially with Dry-ice or other means. The spontaneously self-freezing of the serum cannot be accomplished with the cold condensers of the lyophile apparatus, except with very small quantities, because of the insufficiently rapid removal of water vapor by that means.

The apparatus and Drierite may be purchased from the F. J. Stokes Machine Co., Philadelphia, Pa. In regeneration of the Drierite, with smaller units, the ordinary hot air sterilizing ovens may be used for driving off the moisture. The larger models of the apparatus are equipped with built-in electrical regeneration units so that the chemical need not be removed from the apparatus. When the Drierite has been used to capacity, the electric switch is turned on for regeneration. After a few hours, the material is allowed to cool over night and is then ready for further use.

At no time is Dry-Ice storage required, much less attention is needed, and there is no increased cost resulting from sub-capacity operation. These and other factors, as well as the low general cost, make this process much more practical for clinical laboratory use.

CHAPTER XXVII

METHODS FOR CONDUCTING AGGLUTINATION TESTS

Agglutination methods for the identification and differentiation of pneumococci, meningococci, typhoid, paratyphoid and dysentery bacilli, *B. mallei*, *Vibrio comma*, *Pasteurella boviseptica* and other *Pasteurella* of hemorrhagic septicemia, *Leptospira icterohaemorrhagiae*, etc., are given in Chapter XIX under the respective organisms.

The agglutination tests for human blood grouping are given in Chapter XXVIII.

In this chapter are given methods for agglutination tests with patients' sera submitted for diagnostic purposes.

ROUTINE MACROSCOPIC AGGLUTINATION TEST

1. Obtain 2 to 5 c.c. of patient's blood by venous puncture and separate the serum.

2. Arrange 6 small test tubes in a rack.

3. In the first tube place 2.3 c.c. of 0.85% saline and 1 c.c. in all the remaining tubes.

4. To the first tube add 0.2 c.c. of serum; mix, transfer 1 c.c. to tube No. 2 and discard 0.5 c.c.; mix the contents of tube No. 2 and transfer 1 c.c. to tube No. 3 and so on until the last tube from which 1 c.c. is discarded.

5. To all tubes add 1 c.c. of antigen and shake well. The final dilutions of serum will now be 1:25; 1:50; 1:100; 1:200; 1:400 and 1:800. (*The preparation of the various antigens that may be used are described under the diseases for which this test is recommended.*)

6. Incubate the tubes at 50° to 55° C. for 2 hours and place in the refrigerator overnight.

7. The following *controls* should be included; a set up similar to the above using a positive serum and one with a negative serum. Also include 1 tube carrying 1 c.c. of saline and 1 c.c. of antigen; this is known as the *antigen control tube*.

8. *Readings:* When reading the test, examine the *antigen control tube first*. It should be uniformly cloudy, showing the organisms to be in suspension. If there is any settling at all it should show as a small spot in the bottom of the tube. Examine the negative serum control to eliminate any possible nonspecific or spontaneous agglutination in dilutions other than those considered within the normal limit. The positive control tubes should show positive reactions indicating the sensitivity of the antigen.

The reaction in each tube of the unknown should be noted. A positive reaction is indicated by a clear supernatant fluid with an irregularly distributed granular or

591

flocculent sediment. To examine sediment hold tube rigid at the top and gently tap the bottom just sufficient to stir up the sediment which appears as masses or clumps in positive reactions. Too much agitation may resuspend the antigen. Partial agglutination is recorded when the supernatant fluid is slightly cloudy (less

FIG. 321.—POSITIVE MACROSCOPIC AGGLUTINATION REACTION
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

than in the antigen control tube) and the sediment definitely granular or floccular (Fig. 321).

AGGLUTINATION METHODS FOR TYPHOID AND PARATYPHOID FEVERS

Principles.—1. Many different methods have been proposed. The oldest is the microscopic method of Widal and Grunbaum, employing living cultures and solutions of dried blood or serum.

2. Typhoid and paratyphoid bacilli usually contain two antigens designated as follows: (1) The H antigen occurring in the flagella of motile forms and producing H agglutinin. The antigen is thermolabile and is destroyed by 50% alcohol. The agglutinin, however, is thermostabile and produces *floccular* agglutination (large, coarse, or flaky masses) and is believed to be largely type or species specific. (2) The O antigen occurring in the somata (bodies of the organisms) and producing O agglutinin. The antigen is thermostabile, resistant to 50% alcohol but inhibited by strong solutions of formaldehyde and phenol. The agglutinin is thermolabile and produces the *granular* type of agglutination (fine granular masses) believed to be largely group specific (may agglutinate *B. enteritidis* and other enteric organisms).

3. The use of both H and O antigens in agglutination tests is believed to be of more diagnostic value than microscopic or macroscopic tests employing a single living culture. Thus it appears that normal agglutinins are not as likely to be

present for H antigen as for O antigen. Positive reactions with H antigen (floccular) indicates that the individual has typhoid fever, has had it or has had vaccine. Positive reactions with O antigen (granular) are said to occur earlier in the disease (even with negative blood cultures) and to indicate that the individual has infection caused by the species of organism used in the test or to one closely allied to it. This agglutinin, however, may occur in the sera of normal individuals in final dilutions as high as 1:100.

In individuals immunized with vaccine and showing symptoms of typhoid fever, it is stated that positive O agglutination establishes the diagnosis of typhoid fever, whereas positive H agglutination is without significance.

4. Individuals actively immunized with typhoid-paratyphoid vaccine may show a temporary increase of typhoid agglutinins during undulant fever, influenza and possibly other acute infectious diseases. This is called the *anamnestic reaction* and is frequently confusing when typhoid fever is suspected in individuals who have
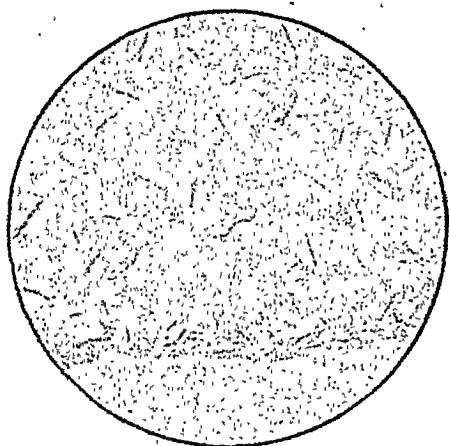
FIG. 322.—A SATISFACTORY CULTURE FOR MICROSCOPE WIDAL TEST

Note proper density and freedom from clumping. (From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)
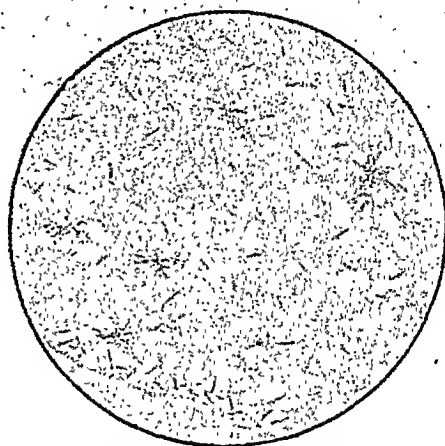
FIG. 323.—AN UNSATISFACTORY CULTURE FOR MICROSCOPE WIDAL TEST

Note that it is too heavy and shows false clumping. (From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

had a previous attack or the vaccine. If typhoid fever is present the agglutinins, however, usually progressively increase and according to Topley and Wilson, especially for the O antigen.

5. Not all strains of typhoid and paratyphoid bacilli are suitable for agglutination tests. Some strains are not agglutinable (due to the presence of the Vi or virulent antigen) and especially those recently isolated. Antigens should be prepared of suitable strains like O901 and H901 which are believed suitable for the preparation of both antigens. Hospital laboratories can usually obtain suitable strains from their State Board of Health laboratories.

6. The temperature and duration of incubation of antigen and serum are likewise important technical phases.

7. Not all cases of typhoid fever give positive agglutination reactions. With microscopic tests employing living cultures positive reactions occur in about 60 to 70% during the first week and 80 to 90% by the third or fourth week. By using both antigens, however, a higher percentage of positive reactions occur.

Microscopic Tests Employing Living Cultures.—1. Actively motile organisms of smooth colonies are preferred. The cultures of typhoid or paratyphoid bacilli (A and B) may be grown in broth for 18 to 24 hours. At 25° C. (on top of the incubator) longer forms usually occur. Do not shake the culture and use upper part. Stock cultures on agar slants may be kept in a refrigerator and transplanted at intervals. When tests are frequently conducted the broth cultures should be subcultured daily. Gilbert and Coleman recommend 2 to 4 hour cultures in bile peptone solution, the density of which is approximately one-half that of No. 1 of the McFarland nephelometer. The culture should be free of clumps or spontaneous agglutination (Figs. 322 and 323).

FIG. 324.—A POSITIVE WIDAL REACTION (Wood)

2. Since living cultures are employed, due care must be exercised and the slides, coverglasses, etc., placed in 5% cresol or boiled for 5 minutes before handling and cleaning. The working table should be wiped with 5% cresol.

3. The tests may be conducted with serum as follows: (a) Prick finger and obtain 0.5 to 1.0 c.c. of blood in a small test tube or fill a Wright's capsule.

(b) Draw off serum with capillary pipet after standing or centrifuging (serum should be clear and free of corpuscles).

(c) Take two small watch crystals, hollow slides, or small test tubes and place 0.05 of serum in each.

(d) Add to one 1.0 c.c. of normal salt solution and to the other 2.0 c.c., making dilutions approximately 1:20 and 1:40 respectively.

(e) Place 1 loopful of culture of typhoid bacilli in the middle of each of 3 coverglasses.

(f) To the first, mix one loopful of serum diluted 1:20. To the second, mix 1 loopful of serum diluted 1:40. To the third, mix 1 loopful of normal salt solution.

(g) Mount each in vaseline on hanging drop slide. The final dilutions obtained are 1:40 and 1:80; therefore, mark the slides as follows: No. 1, 1:40; No. 2, 1:80; and No. 3 control.

(h) Make similar preparations with the paratyphoid cultures.

(i) Allow to stand at room temperature or preferably in an incubator at 37° C. for an hour.

(j) Examine with 1/6 objective using very subdued light. Controls should be inspected first and should not show any clumping or loss of motility.

(*k*) Examine the 1:40 and 1:80 dilutions for clumping and loss of motility and agglutination (Fig. 324).

(*l*) Higher dilutions may be employed but the above are ordinarily sufficient. If the patient has not had a previous attack of typhoid fever or typhoid-paratyphoid vaccine within 3 years, positive reactions are usually indicative of typhoid fever.

Macroscopic Test Tube Method.—1. Prepare the antigen of suitable agglutinable motile strains producing smooth colonies. Inoculate pint Blake bottles of beef infusion agar (pH 7.0 to 7.2) and incubate for 24 hours at 37° C. Wash off with 0.35% formalin in normal saline solution (0.35 c.c. of U.S.P. formalin diluted to 100 c.c.) and dilute with the same until the suspension corresponds in turbidity to No. 3 of the McFarland nephelometer or to contain about 900 million bacilli per c.c. It may be necessary to filter through several layers of cheesecloth to remove large clumps and secure an even suspension. This antigen will keep for at least 6 months in a refrigerator.

Or the antigen may be prepared by cultivating the organism in broth (pH 7.0 to 7.2) for about 72 hours. Centrifuge thoroughly at high speed and discard the supernatant fluid. Suspend the sediment of bacilli in the 0.35% formalin solution and dilute to the same turbidity or numerical strength. Filter through cheesecloth if necessary and keep in refrigerator.

2. Conduct the tests as described on page 591.

3. If the tests are conducted with the serum of an individual previously vaccinated with typhoid-paratyphoid vaccine and suspected of having typhoid fever, the test must be conducted every 2 or 3 days with the same antigen and with great technical care. A definite rise in agglutination titer indicates typhoid infection; if the titer remains fairly constant, typhoid infection is probably not present.

4. Duplicate tests may be set up in the same manner with antigens of the paratyphoid bacilli (A and B) prepared by either method.

Macroscopic Tests with H and O Antigens.—At the present time it may be stated that agglutination tests with these two antigens, one (H) to demonstrate the flagellar or species-specific, and the other, the somatic (O) or group agglutinative properties, usually furnishes information of more diagnostic value than the microscopic test with living culture.

1. The H or *formalin treated antigen* may be prepared as described by Gilbert and her colleagues as follows: the 24-hour growth from beef-infusion agar in a pint Blake bottle is suspended in from 20 to 30 c.c. of 0.85% salt solution containing 0.2% formalin. After 72 hours in the refrigerator, tests for bacterial growth are made and, if necessary, more formalized salt solution is added. After the micro-organisms have been killed, the turbidity is adjusted to correspond to barium sulphate standard No. 3 by the addition of salt solution containing 2.0% formalin. and the agglutinability of the suspension is tested.

2. The O or *alcohol treated antigen* is prepared by washing the growth in 10 c.c. of 0.85% salt solution containing 0.5% phenol. The growth from several bottles is combined and one-half the volume of absolute alcohol or a porportional amount

of 95% alcohol is added slowly, while the suspension is constantly stirred. It is then allowed to remain at a temperature of 35 to 37° C. for about 18 hours after which the supernatant fluid is decanted and tested for bacterial growth and agglutinability. After determination of the dilution necessary to secure a density equivalent to that of barium sulphate standard No. 3, sufficient alcohol is added to the concentrated suspension to give 2.5% in the diluted antigen, which should contain not more than 0.01% phenol.

3. The tests are set up in small test tubes as described on page 591 with each antigen.

4. Clear supernatant fluids and, after gentle shaking, clumps which are definitely seen by the unaided eye, constitute positive reactions. Vigorous shaking should be avoided, since clumps of the flocculate type (II) are easily dispersed.

Absorption Test for Differentiating Between Typhoid and Paratyphoid Fevers.—1. Arrange 4 rows of 4 small test tubes, each row to contain 1 c.c. of serum dilutions 1:20, 1:40, 1:80 and 1:160, respectively.

2. In each tube of the first and second rows emulsify 5 large loopfuls of typhoid bacilli. Add an additional control tube carrying 1 c.c. of saline and culture.

3. In each tube of the third and fourth rows place paratyphoid bacilli (A or B as decided); add a control tube.

4. Mix gently and place in water bath at 37° C. for 2 hours. The results are then recorded.

5. Centrifuge all tubes except controls and transfer supernatant fluids to four more rows of tubes.

6. To each tube of the first and third rows add typhoid bacilli; to the second and fourth rows add paratyphoid bacilli. Mix well and place in water bath 2 hours at 37° C.

7. If typhoid fever is present, agglutination will be strong in the first and second rows of the first part of test and practically unchanged in the third row of the second part.

8. If paratyphoid is present, agglutination will be strong in the third and fourth rows in first part of the test and practically unchanged in the fourth row of the second part. If paratyphoid B is employed with negative results, repeat the test with paratyphoid A.

AGGLUTINATION TESTS FOR BRUCELLA INFECTION OF MAN (UNDULANT FEVER) AND OF THE LOWER ANIMALS

Brucella infection of man resulting in undulant and Malta fevers is likely to be mistaken clinically for typhoid fever. It is therefore a growing and praiseworthy custom to routinely test all sera for *Brucella abortus* agglutinin submitted for the typhoid test. For this purpose the test tube method described below is fairly satisfactory although the Huddleson method is better.

Some laboratories conduct a test on all sera submitted for the Wassermann test since the disease may occur in a clinically unrecognized form because of mild symptoms and particularly in young adults and children.

Test Tube Method with Human and Cattle Sera.—1. The *antigen* may be prepared by cultivating a suitable agglutinable strain of *Br. abortus* on glycerin agar or liver infusion agar in pint Blake bottles for 48 to 72 hours. Wash off with 0.5% phenol in saline solution and shake with glass beads to secure an even suspension. If necessary, filter through several layers of sterile cheesecloth or paper to remove large clumps or pieces of medium and dilute with the phenolized saline to give a suspension corresponding to one-half the density of No. 3 of the McFarland nephelometer.

Or the antigen (second method) may be prepared by washing off the growths with plain saline solution. Shake with glass beads and filter if necessary through cheesecloth or paper. Dilute with saline solution to give suspension corresponding to one-half the density of No. 3 of the McFarland nephelometer. Heat in a water bath at 60 to 65° C. for one hour. To each 100 c.c. add 0.1 c.c. of formalin as a preservative.

Both antigens should be kept in a refrigerator for 2 to 4 days when subcultures are made on liver infusion agar and incubated for 5 to 7 days for sterility. Both antigens will keep at least six months in a refrigerator.

2. In testing *human* sera (unheated) the macroscopic method described on page 591 may be employed.

A positive reaction in 1:100 or higher indicates the probable presence of undulant fever due to infection with *Brucella abortus*. Normal agglutinins may produce partial agglutination at 1:50.

3. In testing *cattle* sera (unheated) arrange 5 test tubes and place the following amounts of serum: 0.1, 0.04, 0.02 and 0.01 c.c. Place 0.1 c.c of saline in No. 5 (antigen control).

(*a*) Add 2 c.c. of antigen to each tube and mix. The final dilutions in the first 4 tubes are now 1:20, 1:50, 1:100 and 1:200.

(*b*) Place in water bath or incubator at 55° C. for 4 hours and then in refrigerator overnight, or place in incubator at 37° C. for 24 hours.

(*c*) Agglutination at 1:50 or higher is a positive reaction. Partial agglutination at 1:50 is weakly positive. Complete or partial agglutination in 1:20 only is suspicious. No agglutination at 1:20 or higher is negative.

Huddleson Rapid Agglutination Test.—The agglutination test by Huddleson and his coworkers described herewith affords a rapid and accurate method for diagnosis with human and bovine sera and is regarded as just as reliable and specific as slower methods; owing to the extremely small size of the organism, microscopic tests place quite a strain on the eyes.

Apparatus.—1. Serological pipets of 0.2 c.c. capacity graduated to 0.01 c.c.

2. Two or 3 plates of double thickness window glass ruled with diamond-point into inch squares. A convenient size is 14 by 6 inches with 12 squares ruled horizontally by 5 squares vertically, leaving a blank margin of one-half inch around the bottom and top of the plate and 1 inch on the ends.

3. Dark-field illumination box (optional). This apparatus, while of unquestion-

able value for making readings, may be dispensed with if the glass plate is placed upon an ordinary laboratory table with a black background.

A convenient size box, 14 inches long by 9 inches wide and 8 inches deep, can be constructed from one-half inch material. One side of the top is covered to a width of 3 inches to protect the eyes of the operator from the lights which are placed just under the top extension piece near the ends. The inside of the box behind the lights is painted white and the remainder black. This serves to produce a black
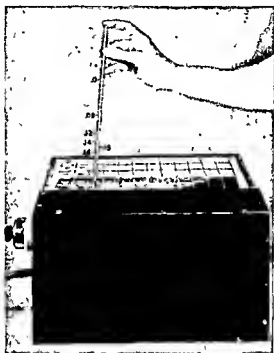
FIG. 325.—ACGLUTINATION TEST FOR UNDULANT FEVER AND ABORTION DISEASE
Placing serum (Huddleson)

background for objects placed on the plate which receive their illumination indirectly. A snap switch on the cord controls the lights (see Fig. 325).

4. Clean toothpicks for mixing serum and antigen on the glass plate.

Preparation of Antigen.—1. Inoculate Blake bottles containing liver infusion agar with a suspension of *Brucella abortus*. A single strain, the agglutinability of which has been previously determined by other tests, should be used.

2. Incubate the cultures for 72 hours at 37° C.

3. Wash off the growth with distilled water containing 12% sodium chloride (C.P.) and 0.5% phenol. Use as little solution as possible or otherwise the suspension will be too dilute and it will be necessary to centrifuge and remove some of the supernatant fluid. It takes much longer to remove the growth with this solution than with ordinary physiological saline (often as long as 2 hours).

4. Filter the suspension through a thin layer of absorbent cotton to remove pieces of the medium or other foreign material.

5. Add 0.01 c.c. of a saturated aqueous solution of gentian violet to each 100 c.c. of suspension. This is added to prevent the growth of organisms not inhibited by the phenol.

6. Place the suspension in a beaker and slowly boil for 10 minutes.

7. Filter through a thin layer of absorbent cotton.

8. Cool rapidly and adjust the reaction to pH 6.8. The antigen is now ready to be standardized.

Standardization of Antigen.—1. Place 0.5 c.c. of antigen in each of 5 small test tubes and mark the tubes from 1 to 5.

2. Add 12% saline solution to each tube as follows:

> No. 1: 0.1 c.c.
> No. 2: 0.2 c.c.
> No. 3: 0.3 c.c.
> No. 4: 0.4 c.c.
> No. 5: 0.5 c.c.

3. Thoroughly mix the contents of each tube.

4. Test each of the 5 dilutions against 3 types of sera, namely, one containing agglutinins in a titer of 1:500 or 1:1000, one with a titer of 1:25, and a negative serum.

5. The dilution which shows an absence of clumping with the negative serum, complete clumping in 0.08 c.c. of the serum with the titer of 1:25, and complete up to and including 0.004 c.c. of the serum with 1:500 titer, is the one properly concentrated for use. The antigen can now be diluted in the same proportion as that in the tube giving the proper reactions in this test.

. **Procedure.**—1. Separate the sera from the clots and centrifuge if necessary to remove corpuscles. Use unheated.

2. *The dark-field box should be placed where it is not too intensely lighted, as too much light from above interferes with the indirect lighting of the plate from inside the box.* A desk lamp is of great advantage while the serum and antigen are being placed on the plate but it should be turned off when the tests are ready to be read so as not to interfere with the indirect lighting from the box. *Do not turn on the light in the box until ready to read the test as the plate will otherwise become too warm.*

3. Arrange the serum samples in a row parallel with the box. The glass plate, with the etched squares upward, is placed over the opening of the box and the identification number of the serum sample marked with a wax pencil on either the top or bottom of the row of squares used.

4. With a clean 0.2 c.c. pipet, draw up serum from the first blood sample to the zero mark on the pipet. Beginning in the bottom left-hand square of the plate, place the following amounts of serum in the succeeding squares towards the top (see Fig. 325):

Reading on Pipet

1st square (0.08 c.c.)	0.08
2nd square (0.04 c.c.)	0.12
3rd square (0.02 c.c.)	0.14
4th square (0.01 c.c.)	0.15
5th square (0.004 c.c.)	about midway, 0.15 and 0.16

5. This manner of placing the serum brings the smallest amount farthest from the heat of the electric bulb, *reducing the rapidity of drying of the smallest amounts of serum.* The procedure is continued, using the next set of vertical squares and a separate pipet for each sample. The best results are obtained by testing only four or five samples at a time, as otherwise the small amounts of serum dry out too much before the test is completed.

6. If the pipet has been placed deep in the serum, there will be some serum which will collect on the outside at the tip. For accuracy, this should be removed by touching the tip of the pipet against the lip of the vial.

7. After thoroughly shaking the vial, remove a dropper full of the antigen. Holding the dropper in a vertical position, add one drop to each amount of serum on the plate. *Care should be taken to hold the*

Fig. 326.—Agglutination Test for Undulant Fever and Abortion Disease
Adding antigen (Huddleson)

dropper in a vertical position since holding it at another angle will make a considerable difference in the amount of antigen delivered. Always replace the dropper directly in the vial of antigen (Fig. 326).

8. The final dilutions are now 1:25, 1:50, 1:100, 1:200 and 1:500.

9. With a clean toothpick mix the serum and antigen, using a new toothpick for each sample. *Always start at the top of the plate in the square containing the smallest amount (0.004 c.c.) of serum and continue downward to the largest amount.* Spread the mixture over about three-fourths the area of the square without coming in contact with the etched dividing lines.

10. Immediately after the samples have been mixed, remove the plate from the box and tilt slightly backward and forward slowly for about two minutes. Place the plate on the box, turn on the light and record the results. When working without the box, make the readings against a light so that the plate is illuminated from beneath.

11. The reactions stand out very clearly as shown in Figure 327. It is not

difficult to distinguish between complete clumping of the antigen and different degrees of incomplete clumping, *e.g.*, in sample 6 there is complete agglutination in all amounts of serum, while in sample 4 the clumping is complete in only the last three amounts. A negative serum (sample 1) causes no flocculation of the antigen. There are often encountered, however, sera which produce a trace of flocculation in the 0.08 c.c. amount. In the "slow" or test tube method, this occurrence will often pass unobserved unless viewed with a hand lens. This type of clumping appears to be due to the presence of native agglutinins in the serum.

12. Immediately after using, the pipets should be rinsed several times with fresh water until thoroughly clean. Then boil in distilled water and drain all the water out before using again. The glass plate may be cleansed with cleaning powder and brush, after which it is rinsed with distilled water and dried. By having several clean plates available, one can proceed with the testing of additional samples without delay. *Absolute cleanliness of glassware is essential.*

Fig. 327.—Different Degrees of Agglutination Reactions for Undulant Fever and Abortion Disease (Huddleson)

Interpretation.—1. With human sera a positive reaction in a titer of 1:100 is considered diagnostic of undulant fever.

2. With cattle sera a positive reaction in a dilution of 1:50 is suspicious of present or past infection, while positive reactions in 1:100 or higher are diagnostic.

3. Agglutinins generally appear in the blood after the infection has been present for a week or longer.

Method for Testing Milk.—This test can be conducted with milk from cows for the detection of those carrying *Brucella abortus* in the udders.

1. Collect separate samples of the fore milk from each quarter, using clean, sterilized vials of about one-half ounce capacity. Before collecting the samples, place in each vial a small amount of rennin powder (about what can be picked up between the prongs of a small pair of forceps or on the tip of a small knife blade). Then draw the milk directly into the vial and place in a slanting position. The whey will separate out in about an hour at room temperature.

2. The test is conducted with the whey in exactly the same manner as previously described for blood sera.

AGGLUTINATION TEST FOR TULAREMIA

1. Prepare the antigen as follows:

(a) Use Blake bottles containing blood glucose cystine agar. Before inoculation, the bottles should dry several days in an inverted position in the incubator and any water of condensation present should be pipeted off. Inoculate each bottle with the entire growth from a blood glucose cystine agar slant of *B. tularense* No. 38, suspended in 1 c.c. of physiological salt solution. Spread the suspension quickly over the surface of the medium in the Blake bottles by rocking them.

(b) Incubate the bottles in their normal noninverted position at 37° C. for 3 days.

(c) Take off the growth of each bottle in 15 c.c. of physiological salt solution containing 0.2 or 0.3% of formalin (U.S.P.) Throw down the bacterial mass by centrifugation, thereby washing the organisms. Pour off the supernatant fluid. Take up the bacterial mass in a small amount of formalized salt solution. This concentrated stock suspension has been found to be entirely reliable for agglutination tests even after storage for 2 years in the cold room.

(d) At the time of use dilute a portion of the concentrated suspension to the desired turbidity corresponding to No. 3 of the McFarland nephelometer.

2. In a series of 7 small test tubes place 0.5 c.c. amounts of the following dilutions of serum: 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. In No. 7 place 0.5 c.c. of saline solution (control).

3. To each tube add 0.5 c.c. of antigen. The final dilutions are now 1:20 to 1:640.

4. Mix well and incubate at 55° C. for 2 hours; place in refrigerator overnight when the final readings are made.

5. It is advisable to set up duplicate tests with antigens of *B. typhosus* and *Br. abortus*.

6. Agglutination in final dilution of 1:80 or higher, is considered diagnostic of tularemia providing there is no cross-agglutination with *Br. abortus*.

AGGLUTINATION TEST FOR GLANDERS OF HORSES, MULES AND MAN

Prepare the antigen as follows with an agglutinable strain of *B. mallei*. Only a small percentage of cultures are suitable.

(a) Cultivate on glycerin agar with pH 6.8 for 24 to 48 hours.

(b) Wash off with sterile saline solution. Shake well to break up clumps. Dilute to density of No. 3 of the McFarland nephelometer. Heat at 65° C. for one hour. Add formalin to give a final 0.25%. Keep tightly stoppered in a refrigerator.

With the sera (unheated) of *horses* and *mules* proceed as follows:

1. For each serum arrange 6 small test tubes and place 1 c.c. of saline in each.

2. Prepare a 1:50 dilution of serum (0.1 c.c. + 4.9 c.c. saline) and place 1 c.c. in No. 1. Mix well and transfer 1 c.c. to No. 2; mix and transfer 1 c.c. to No. 3; mix and discard 1 c.c. Prepare a 1:250 dilution (1 c.c. of 1:50 + 4 c.c. saline) and place 1 c.c. in No. 4; mix and transfer 1 c.c. to No. 5; mix and discard 1 c.c.

The dilutions are now 1:100, 1:200, 1:400, 1:500 and 1:1000. The sixth tube is the control.

3. Add 1 c.c. of antigen to all tubes, which doubles the dilutions (1:200 to 1:2000).

4. Mix and incubate at 37.5° C. for 24 to 48 hours.

5. The reactions are interpreted as follows:

      Positive: complete agglutination 1:1000 or higher

      Suspicious: agglutination in 1:800

      Negative: no agglutination or partial agglutination 1:200 and 1:400

Agglutination Test for Glanders of Man.—The test is not as reliable as the complement-fixation test; the sera of normal persons may have a titer of 1:100 but in acute cases has been observed to reach 1:1000 or higher. On the other hand, it falls very low when the disease reaches the chronic stage and agglutination may not occur at all.

AGGLUTINATION TEST FOR PLAGUE

Agglutinins do not appear until about the ninth day of the disease, thus rendering the agglutination test of little value in early diagnosis but sometimes of confirmatory value in the convalescent state. The agglutination test with high-titer agglutinating sera is, however, of value for identifying cultures.

1. The *antigen* may be prepared by washing off growths on agar with 0.5% phenol in normal saline solution. Shake with glass beads to secure an even suspension and dilute with the phenolized saline to match No. 3 of the McFarland nephelometer. Place in incubator for 24 hours and then in refrigerator for 2 to 4 days. Culture for sterility. There is a tendency to spontaneous agglutination.

2. Arrange a series of 7 small test tubes. In No. 1 place 0.8 c.c. of saline and 0.5 c.c. in the remaining six.

3. Use the serum unheated and place 0.2 c.c. in No. 1. Mix well and transfer 0.5 c.c. to No. 2; mix well and transfer 0.5 c.c. to No. 3 and so on to No. 6 from which 0.5 c.c. is discard. No. 7 is the antigen control.

4. To each tube add 0.5 c.c. of antigen. The final dilutions in the first 6 tubes are now 1:10 to 1:320.

5. Mix well and place in water bath or incubator at 55° C. for 4 hours when the readings are made.

6. The organism displays a strong tendency to auto-agglutination. Examine the control very carefully.

7. Positive agglutination in final dilutions of 1:10 to 1:50 or higher is regarded as diagnostic.

AGGLUTINATION TEST FOR TYPHUS FEVER (WEIL-FELIX REACTION)

Principles.—1. After the fourth day of typhus fever there usually develops in the serum agglutinins which react with certain strains of Proteus organisms, which though isolated from typhus cases are presumable without etiological relationship to the disease.

2. The strain usually employed is Proteus X19 of Weil and Felix. However, this strain is not always agglutinated by the sera of typhus patients and it is desirable to also use the "Kingsbury" strain.

Technic.—1. For antigen it is best to use a suspension of living cultures. They are grown on slants of agar (pH 7.4) prepared of fresh meat and sterilized at 100° C. The culture should be first streaked on plates of agar, incubated at 37° C. for 24 hours and smooth, nonspreading, nonmotile or "O" colonies selected for the preparation of antigen (OX19). If "H" antigen is desired, select colonies of motile bacilli and prepare with alcohol as described for the preparation of "H" antigen of typhoid bacilli.

Culture on agar for 24 to 48 hours and wash off with sterile normal saline solution. Shake with glass beads to secure an even suspension and dilute with saline to correspond in turbidity to tube No. 3 of the McFarland nephelometer.

2. Conduct a macroscopic test as described on page 591.

3. At the same time set up duplicate tests, employing a known positive human or rabbit immune serum; also testing a known normal human serum.

In typhus fever, positive agglutination usually occurs from 1:50 to 1:50,000. In the majority of cases the titer is about 1:25 on the fourth day and 1:50 or higher by the eighth day. By the end of the second week it may reach several thousands, after which it declines rapidly during convalescence. The reaction may be negative within five or more months after recovery, or may persist for several years. Zone reactions may occur with no agglutination in the lower dilutions.

The titer of normal sera is rarely higher than 1:25 to 1:50. Agglutination at 1:50 may be significant, however, if following a previous negative reaction.

Some confusion has arisen with the reaction in differentiating typhus fever from certain typhus-like infections. The following table after Felix (*Trans. Roy. Soc. Trop. Med.*, 1933, 27: 147) may be helpful:

Disease	Locality	Agglutination			Main Antigen
		OX19	OX2	OXK	
Classical typhus .	Old and New Worlds	+++	+	—	OX19
Tabardilla; Brill's disease	Mexico; U. S. . .	+++	?	—	OX19
Endemic typhus .	Australia . .	+++	+	—	OX19
Tropical typhus (Shop)..	Malaya; Dutch East Indies	+++	+?	—	OX19
Tropical typhus (type K)...	Malaya; Dutch East Indies....	—	—	+++	OXK
Tsutsugamushi...	Japan . . .	—	—	+++?	OXA(?)
Rocky Mountain Spotted fever	U. S . . .	+	+	+	Unknown
Tick-bite fever	South Africa	+	+	+	Unknown
San Paulo typhus	Brazil	+	+	+	OX19

+++ = main agglutination, + = group agglutination, ? = not tested adequately.

AGGLUTINATION TEST FOR BACILLARY DYSENTERY

Agglutination tests do not possess very much diagnostic value because of the frequency of negative reactions. If employed, 2 antigens are required prepared of

the Shiga and Flexner strains of *B. dysenteriae* as these do not possess identical agglutinable properties.

A macroscopic test tube method is preferred as described on page 591. The antigens may be prepared by the same technic as for the macroscopic agglutination test for typhoid fever.

According to most observers, normal human serum never agglutinates dysentery bacilli in dilutions greater than 1:25. With the Shiga strain agglutination should occur at 1:50 to 1:100 to be significant and the Flexner strain at 1:100 to 1:200.

AGGLUTINATION TEST FOR PERTUSSIS

Agglutination tests for *pertussis* have not proved of much diagnostic value. Normal serum may agglutinate in final dilutions up to 1:100. The antigen should be prepared of the bacillus in phase I. It may be cultivated on plates of the Bordet-Gengou medium and washed off with saline solution. Shake thoroughly with glass beads and filter through cotton to secure an even suspension. Dilute with saline to match tube No. 3 of the McFarland nephelometer. Add merthiolate to a final concentration of 1:10,000 for sterility and as a preservative. Set up macroscopic tests in test tubes with final dilutions of 1:100, 1:200, etc. Incubate at 55° C. for 4 hours and place in the refrigerator overnight when the readings are made.

AGGLUTINATION TEST FOR BACILLARY WHITE DIARRHEA OF CHICKENS

1. Cultivate *Salmonella pullorum* on agar for forty-eight hours. Wash off with phenolized saline solution. Shake well and filter through cotton. Dilute to proper density. To each 100 c.c. add 2 c.c. of .2% solution of sodium hydroxide as recommended by Matthews for the prevention of precipitation, giving the cloudy reactions which may occur with as high as 75% of sera.

2. The sera should be clear and as free as possible of hemoglobin.

3. For the test arrange 4 small test tubes.

4. Place 1.8 c.c. of saline in No. 1 and 1 c.c. in Nos. 2, 3, and 4.

5. To No. 1 add 0.2 c.c. of serum; mix and transfer 1 c.c. to No. 2; mix and transfer 1 c.c. to No. 3; mix and discard 1 c.c. No. 4 receives no serum and is the antigen control.

6. Add 1 c.c. of antigen to all tubes to give final dilutions of 1:20, 1:40 and 1:80.

7. Mix well. Incubate at 37.5° C. for 24 hours.

8. The results are interpreted as follows:

   Positive: agglutination in 1:40 or higher

   Suspicious: partial agglutination in 1:20

   Negative: no agglutination in 1:20

AGGLUTINATION TEST FOR FOWL TYPHOID

1. Cultivate *Salmonella gallinarum* (*B. sanguinarium*) on agar for 2 or 3 days. Wash off the growths with phenolized saline solution and shake well. Filter

through cotton and dilute to proper density. To each 100 c.c. may be added 2 c.c. of 2% solution of sodium hydroxide for aid in preventing the precipitation sometimes yielding the cloudy reactions observed with fowl serum.

2. The sera should be fresh and as free as possible of hemoglobin.

3. For each serum arrange 6 small test tubes. Into No. 1 place 2.4 c.c. saline and 1 c.c. in the remaining tubes.

4. To No. 1 add 0.1 c.c. of serum. Mix, transfer 1 c.c. to No. 2 and discard 0.5 c.c. Mix No. 2 and transfer 1 c.c. to No. 3; mix and transfer 1 c.c. to No. 4; mix and transfer 1 c.c. to No. 5; mix and discard 1 c.c. No. 6 is the antigen control and receives no serum.

5. To each tube add 1 c.c. of antigen. The final dilutions are now 1:50, 1:100, 1:200, 1:400 and 1:800.

6. Mix well and incubate at 37.5° C. for 21 hours.

7. The results are interpreted as follows:

      Positive: agglutination 1:200 or higher

      Suspicious: partial agglutination in 1:50 to 1:100 but not higher

      Negative: no agglutination 1:50 or higher

CHAPTER XXVIII

METHODS FOR CONDUCTING PRETRANSFUSION BLOOD TESTS

Principles.—1. The serum of a patient (recipient) may agglutinate or hemolyze the corpuscles of a donor or the corpuscles of the recipient may be agglutinated or hemolyzed by the serum of a donor. Blood transfusion requires, therefore, the use of compatible blood. It was found recently that it is necessary to select persons with identical blood groups for the injection of blood with plasmodium of malaria into recipients with general paresis and preliminary to skin grafting operations. Hemolysis does not occur without agglutination and since the latter is more easily detected it is sufficient to test only for agglutination.

2. This phenomenon is due to the fact that there are two main agglutinins (*a* and *b*) occurring in the serum and two main agglutinogens (A and B) occurring in the red blood corpuscles of human beings which permit placing the blood of an individual in any one of four main groups. Considerable confusion, however, and even accidents, have been caused by different arbitrary numbers or classifications, the three best known being as follows:

Moss	Jansky	Landsteiner (International)	Percent of Approximate Occurrence in Adults in the United States *
I	IV	AB	4
II	II	A	39
III	III	B	12
IV	I	O	45

* These figures are based on a study of 38,000 persons in different parts of the United States quoted from A S Wiener, *Blood Groups and Blood Transfusions*, Charles C Thomas, 1935, p 158

3. If both agglutinogens are present in the corpuscles the serum is free of both agglutinins and the individual belongs to group AB or Moss I or Jansky IV. If the corpuscles contain neither agglutinogen the serum contains both agglutinins and the blood belongs to O or Moss IV or Jansky I. If the corpuscles contain agglutinogen A, the serum contains agglutinin *b* and the blood belongs to group A. Moss II or Jansky II. If the corpuscles contain agglutinogen B, the serum contains agglutinin *a* and the blood belongs to group B. Moss III or Jansky III. In other words, the serum regularly contains the agglutinins active for the absent agglutinogens; that is, corresponding agglutinins and agglutinogens do not coexist in one blood.

PRECAUTIONS AND SOURCES OF ERROR

1. Although pretransfusion blood tests are in themselves relatively simple procedures, their performance requires *a high degree of individual and group responsibility*. In each laboratory where these tests are made there should be a standard routine procedure designed especially to eliminate mistakes and prevent accidents. Extreme care should be exerted to avoid the mixing up of specimens, etc. All test tubes and slides should be labeled, the tubes containing serum and cell suspensions of the same individual should be kept adjacent; recipient's blood may be kept in slightly longer tubes.

2. It is to be especially noted that group I of Moss corresponds to IV of Jansky and that IV of Moss corresponds to I of Jansky. If blood grouping is reported by numbers, it is essential therefore always to state whether the Moss or Jansky classification is being used, in order to avoid serious accidents. It is still better to drop both of these and to employ exclusively the Landsteiner classification which is recognized officially as the International nomenclature.

3. For blood transfusion the recipient's blood should be typed and a donor selected belonging to the same group. But direct matching is recommended in addition, due to the existence not alone of subgroups and atypical agglutinins, but also of donors with unusually high agglutinin titers. Such high agglutinin titers may be dangerous when a so-called universal donor O (I Jansky, IV Moss) is used. However, it is not quite safe to depend on direct matching alone since in cases of low titer agglutinins, incompatibility may escape attention. In other words, if only one of the two methods for selecting donors is to be depended on direct ' matching is preferable, but it is better and therefore recommended that the blood of the recipient be typed and direct matching tests conducted with the blood of donors belonging to the same group for the final selection of a donor.

4. Preferably, tests should be done at a room temperature of 70° to 80° F. to avoid errors due to "cold" or auto-agglutinins which may become operative at low temperatures. If reagents (test serums) are kept in the ice-box, care should be taken that room temperature be approximated before using them. Serum with "cold" or auto-agglutinins will agglutinate cells of all blood groups, even those of the same individual. Such agglutinins can be removed by separating serum from the cells at ice-box temperature (0°—5° C.).

5. Pseudo-agglutination is a possible source of error readily avoided by dilution.

6. In microscopical tests, rouleaux formation of corpuscles is a possible source of error, especially for inexperienced workers. It is favored: (a) by high concentration of sera and, (b) by high temperature. It is best avoided by diluting the serum 1:2 or 1:3 with saline. Sometimes it is sufficient to add a small drop of saline to the slide preparation.

7. A more important source of error lies in the fact that sera differ in their agglutinating titer from time to time. Sera with high titers frequently have considerable amounts of isohemolysin. This may occasionally mask agglutination and lead to disastrous accidents. If such a preparation is examined by one unexperi-

enced the absence of clumps (without regard for evidence of hemolysis) may suggest compatibility while the opposite is true. By keeping such a possibility in mind and comparing the slide with the control (cells and saline), the true nature of the phenomenon can easily be recognized. Especial care should be exercised in typing and matching the blood of those who have repeatedly received transfusions. Recipients receiving repeated transfusions may undergo obscure changes and especially require very careful direct matching tests for the selection of donors.

8. Especial care must be exercised in matching the blood of infants under two years, due to uncertainty in the time of appearance of permanent iso-agglutinins.

9. Obviously only healthy donors should be chosen. Those with fever or anemia should not be used. *Syphilis and malaria should be excluded.*

10. It is a wise rule to preserve blood specimens for rechecking if necessary.

METHODS FOR DIRECT MATCHING

Microscopic Agglutination Tests.—1. Prepare a finger or an ear lobe of recipient and make deep puncture.

2. Collect 2 to 4 drops of blood in a small test tube containing 2 c.c. of 1% solution of sodium citrate in normal saline solution. More drops of blood will be needed if the recipient is very anemic.

3. At the same time secure 1 to 2 c.c. of blood in a dry test tube.

4. Secure blood from the prospective donor or donors in the same manner.

5. Break up the clots and centrifuge along with the tubes containing the citrated blood.

6. Remove the supernatant fluids from the citrated tubes and add to the corpuscles in each tube 2 c.c. of normal salt solution; suspend the corpuscles. This makes a 2 to 5% suspension. Remove the sera which should be free of corpuscles.

7. Hanging drop preparations are prepared as follows:

(a) With a large platinum loop, 2 loopsful of recipient's serum plus 1 loopful of donor's corpuscles are mixed on a coverglass and mounted with vaselin on a hanging drop slide.

Fig. 328.—FALSE AGGLUTINATION

(From Kolmer. *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

(b) Two loopsful of donor's serum plus 1 loopful of recipient's corpuscles are mixed and mounted.

(c) Prepare similar preparations with the sera and corpuscles of additional donors.

(d) Two loopsful of saline, and 1 loopful of recipient's corpuscles are mixed and mounted (control).

(e) Two loopsful of saline plus 1 loopful of donor's corpuscles are mixed and mounted (control).

(f) Be sure to label the slides and do not trust to memory.

8. Allow to stand for 15 minutes at room temperature.

9. Examine with the low power objective. Controls should be examined first (step 7, d and e) and should not show any agglutination. False clumping and

Fig. 329.—Rouleaux Formation

(From Wiener, *Blood Group and Blood Transfusion*, Chas. Thomas, Springfield, Ill.). ×400.

rouleaux formation at the margin of the mixture should not be mistaken for positive reactions (Figs. 323, 329, 330 and 331).

10. Agglutination in (a) of step 7 shows that the recipient's serum is incompatible with the donor's corpuscles. Agglutination in (b) shows that the donor's serum is incompatible with the recipient's corpuscles.

11. *This direct test is always advisable before transfusion even if the recipient is first grouped and a donor of the same group is selected* in order to guard against the possibility of transfusing with an incompatible subgroup blood.

An alternative method for setting up the above test is as follows:

1. Prepare capillary pipets of approximately equal caliber for the corpuscles and serum of the recipient and each donor.

2. Arrange 2 clean slides for each test and proceed as follows:

(a) At the end of slide No. 1 mix a drop of the recipient's corpuscles with a drop of donor's serum and a drop of saline solution. At the other end mix a drop

of the recipient's corpuscles with a drop of saline solution (this is a control on the corpuscles).

(*b*) At the end of slide No. 2 mix a drop of the donor's corpuscles with a drop of the recipient's serum and a drop of saline. At the other end mix a drop of the donor's corpuscles with a drop of saline solution (control).

(*c*) Repeat the set-up in the same manner with the corpuscles and serum of each donor.

3. Rock the slides occasionally for about 5 minutes. If agglutination is not visible, place a coverglass over each mixture and let stand at room temperature for 15 minutes. Examine each microscopically.

Macroscopic Agglutination Test (Brice).—1. This test may be used for more rapid testing of large numbers of donors and is conveniently conducted with a special plate (Fig. 332).

2. Corpuscles and sera of the patient (recipient) and each donor are prepared as described above.

3. Mix 1 drop of the recipient's corpuscles with 4 drops of the serum of each of the donors, and a drop of each of the donors' corpuscles with 4 drops of the serum of the recipient, with a

Fig. 330.—A Negative Reaction

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

glass stirring rod, each being in its proper position upon the typing plate.

4. Lift the plate and oscillate with a rocking motion, which keeps all of the corpuscles in movement. for 7 minutes over a white background or transmitted light for evidences of agglutination.

5. The microscopical tests are more sensitive and to be preferred.

Macroscopic Agglutination and Hemolysis Tests (Kolmer).—1. Collect blood in citrate solution and in a small dry test tube from the recipient and donor as described.

2. Prepare the corpuscles and secure the sera as described. Only fresh sera can be used.

3. Arrange 4 small test tubes and place 1 c.c. of saline in each.

4. Into No. 1 place 5 drops of recipient's serum and 1 drop of donor's corpuscles.

5. Into No. 2 place 5 drops of donor's serum and 1 drop of recipient's corpuscles.

6. Into No. 3 place 1 drop of recipient's corpuscles (control).

7. Into No. 4 place 1 drop of donor's corpuscles (control).

8. Mix each tube and place in a water bath at 37° C. for 1 hour.

9. Read the results. Tubes 3 and 4 should show no hemolysis or agglutination.

10. If hemolysis and agglutination do not occur in tubes 1 and 2 the bloods

are compatible. If the recipient's serum is incompatible with the donor's cor-
puscles, a positive reaction will occur in tube No. 1; if the donor's serum is in-
compatible with the patient's corpuscles, a positive reaction will occur in tube
No. 2

FIG. 331.—TRUE AGGLUTINATION

(From Kolmer, Infection, Immunity and Biologic Therapy, W. B. Saunders)

11. Repeat in same manner with the serum and corpuscles of each
12. This test is recommended especially for hemolysis and r
work.

METHODS FOR BLOOD GROUPING

Method of Landsteiner.—1. Secure a few drops of blood i
0.9% solution of sodium chloride to give a 2 to 5% blood suspe
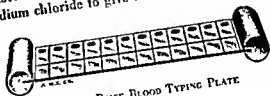
FIG. 332.—BRICE BLOOD TYPING PLATE

2. Into a small test tube (7 millimeters diameter) place a
serum, a drop of saline solution, and a drop of the blood sus
3. Prepare a second tube in the same manner with a drop

   several times and allow to stand a

5. Remove a drop of each mixture with a thin glass rod to glass slides and examine microscopically with low magnification.

6. Positive reactions generally occur within a few minutes but in order to detect unusually feeble reactions, the negative reactions should be examined after 1 hour. Control tests with known cells A and B should be included.

7. The readings and interpretations are made as described above.

Second Microscopic Method.—1. Prepare finger and make puncture of the individual to be grouped.

2. Collect 2 or 3 drops of blood in small test tube containing 1 c.c. of normal salt solution (approximately a 5% suspension).

3. Prepare four capillary pipets of equal calibers, one each for group A (II) serum, group B (III) serum, corpuscle suspension and saline solution.

4. Arrange 3 clean slides.

5. On one slide, place a drop of A (II) serum and add a drop of corpuscles and saline solution; mix well.

6. On the second slide. place a drop of B (III) serum and add a drop of corpuscles and saline solution; mix well.

7. On the third slide mix a drop of corpuscles with a drop of saline solution (control).

8. Rock each slide occasionally for about 5 minutes to accelerate agglutination.

9. Place a coverglass on each and let stand at room temperature for 15 to 20 minutes.

10. Examine under microscope with low-power objective.

11. The control should show no agglutination.

12. The group is determined as follows:

(*a*) No agglutination with either groups A (II) or B (III) serum = group O of Landsteiner, group I of Jansky or group IV of Moss.

(*b*) Agglutination with both groups A (II) and B (III) serum = group AB of Landsteiner, group IV of Jansky. or group I of Moss.

(*c*) Agglutination with group A (II) but no agglutination with group B (III) serum = group B of Landsteiner or group III of both Jansky and Moss.

(*d*) Agglutination with group B (III) but not with group A (II) serum = group A of Landsteiner. or group II of both Jansky and Moss.

13. False clumping and rouleaux formation at the margin of the mixture should not be mistaken for positive reactions.

Sources of Error.—In addition to the sources of error previously mentioned, there are some specific sources of error in the determination of blood groups.

1. The use of low-titered test serum may cause a failure to recognize certain cases belonging to group AB (IV Jansky, I Moss) and to mistake them for the group B (III).

2. The use of fresh high titered test serum may. on account of unrecognized hemolysis. lead to a mistaken diagnosis of group O (I Jansky. IV Moss) instead of group A (II), B (III) and AB (IV Jansky, I Moss). This can be avoided best by using only stored test sera.

3. Test sera must be carefully checked with corpuscles of several persons with known blood groups to exclude the possibility of the presence of irregular agglutinins (mainly cold agglutinins).

Method for Preparing Type Sera.—Known group A (II) and group B (III) sera should be kept in a refrigerator preserved with chloroform or 0.5% phenol. Preservation as well as distinctive coloring of the test sera is achieved by the following procedure (Rosenthal) [1]: To each c.c. of group A (II) serum is added 0.01 c.c. each of 1% aqueous solution of neutral acriflavine and 0.5% aqueous solution of basic fuchsin; and to each c.c. of group B (III) serum is added 0.01 c.c. of 1% aqueous solution of brilliant green. The sera should be preferably from healthy middle-aged individuals of such potency as to produce marked agglutination in 1 minute in final dilution of 1:8 or higher. It is well to replenish every 2 or 3 months and frequent tests should be made to make sure that the sera are potent. It is easy to replenish the sera by typing the corpuscles of blood specimens submitted for the Wassermann test. If corpuscles belonging to group A (II) are found the excess serum may be preserved (group A or II); if corpuscles belonging to group B (III) are found, the excess serum should be preserved (group B or III). These two sera suffice for grouping by the Landsteiner, Moss or Jansky classifications. Some authors recommend the use of type O serum (group IV Moss or I of Jansky) as a check. The cells of all blood groups except the O are agglutinated by that serum. A loss of titer of the A or B typing sera can be detected by the use of O serum.

MEDICOLEGAL APPLICATION OF BLOOD GROUPS FOR EXCLUSION OF PATERNITY

The application is based on two generally accepted laws:

1. The agglutinogens A and B cannot appear in the blood of a child unless present in the blood of one or both parents.

2. A parent belonging to group AB cannot beget a group O child and a group O parent cannot beget a group AB child.

When the blood groups of parents are known, the possible and impossible combinations of blood groups of the children of these unions is shown in table [2] on page 615.

Precautions.—The methods of blood grouping are the same as those described, but it is necessary to determine the group of the red corpuscles and the iso-agglutinins in the serum of every individual. The latter is done by testing the serum with the red corpuscles of persons whose blood groups are known. Furthermore, proper and adequate control tests should be carried out. Careful protocols recording every step must be kept. It is recommended to take photographs or finger prints of every person at the same time when blood samples are obtained as a means of identification.

[1] J. Lab. & Clin. M., 1931, 16:1123.

[2] A. S. Wiener, Blood Groups and Blood Transfusions, Charles C. Thomas, 1935, p. 191.

Groups of Parents	Groups of Children Possible	Groups of Children Not Possible
1. O,O	O	A.B,AB
2. O,A	O.A	B,AB
3. O,B	O,B	A,AB
4. A,A	O,A	B,AB
5. A,B	O,A,B,AB	
6. B,B	O,B	A,AB
7. O,AB	A,B	O,AB
8. A,AB	A,B,AB	O
9. B,AB	A,B,AB	O
10. AB,AB	A,B,AB	O

M and N Agglutinogens.—The examination of the M and N blood types raises considerably the chances of excluding paternity. However, the technical difficulties connected with the production of proper immune sera that are necessary for the carrying out of such tests, limit their application to experts who are thoroughly familiar with all possible mistakes. The corresponding agglutinins do not occur in human blood and must be produced in rabbits. Therefore they are of no importance in relation to pretransfusion tests.

CHAPTER XXIX

METHODS FOR CONDUCTING COMPLEMENT-FIXATION TESTS FOR SYPHILIS AND OTHER DISEASES

KOLMER COMPLEMENT-FIXATION TEST FOR SYPHILIS [1]

Glassware and Apparatus.—1. Pipets (Fig. 333).

> 1 c.c. graduated in 0.01 c.c. to tip.
>
> 5 c.c. graduated in 0.10 c.c.
>
> 10 c.c. graduated in 0.50 c.c.

An automatic pipet is highly recommended for rapid work and saving of time for pipeting saline solution, complement, antigen and hemolysin in the conduct of the tests.

2. Test tubes: 85 by 13 millimeters (inside diameter) with rounded bottoms and no lips.

3. Cylinders: glass-stoppered, graduated (50 or 100 c.c. capacity) to be used for measuring amounts over 50 c.c.
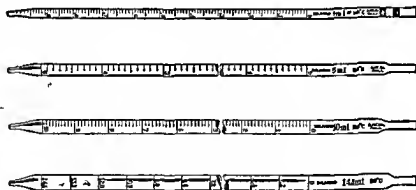
FIG. 333.—KOLMER SEROLOGICAL PIPETS

4. Test tube racks: galvanized wire racks carrying 12 rows of 6 tubes each (Fig. 334).

5. Water bath: any easily regulated water bath is suitable, or a simple gal-

[1] For a more detailed description of this test, including complement-fixation tests for the identification of sera, blood stains, seminal stains and for the detection of meat and milk adulteration, consult Kolmer's *Serum Diagnosis by Complement Fixation*, Lea and Febiger, Philadelphia, 1928. Antigens and hemolysins for the syphilis and other complement fixation tests may be purchased from the Research Institute of Cutaneous Medicine, 2101 Pine Street, Philadelphia.

vanized pan carrying water to a depth of 8 centimeters can be used satisfactorily at 55° or 37° C. (Figs. 335 and 336).

6. Refrigerator: any refrigerator maintaining a temperature of 6° to 8° C. is satisfactory.

Method of Cleaning Glassware.—1. All glassware should be chemically clean and preferably sterile. To clean tubes and flasks, empty and rinse in running tap

FIG. 334.—KOLMER RACK
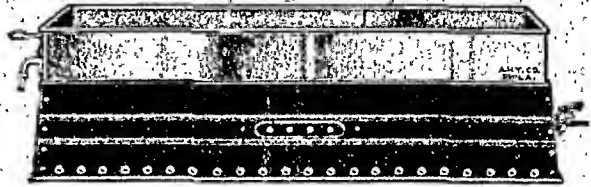
FIG. 335.—KOLMER SEROLOGICAL BATH, GAS HEATED

water; wash inside and outside in soapy water; rinse several times in running tap water and invert in wire baskets. Dry in the hot air oven at about 160° C.

2. Pipets should be placed after use in a jar or cylinder of clean water with a pad of cotton in the bottom. To clean pipets, rinse thoroughly in running tap water, place in a metal box or wire basket and sterilize in the oven.

3. Flasks should be plugged with cotton and sterilized in the oven for 30 minutes at 160° C.

4. If glassware becomes cloudy, immerse in bichromate cleaning fluid (2 parts potassium bichromate, 3 parts commercial sulphuric acid, and 25 parts water) for 24 hours. Rinse thoroughly in running tap water and proceed with the washing as described.

Preparation of Saline Solution.—Dissolve 8.5 grams of dry, chemically pure sodium chloride in 1000 c.c. of tap or
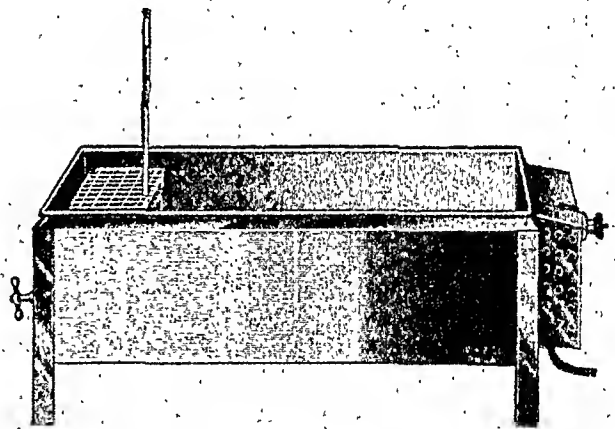
FIG. 336.—KOLMER SEROLOGICAL BATH, ELECTRICALLY HEATED

distilled water (former preferred in most localities). If the salt has absorbed moisture it should be dried in the hot air oven for 10 or 15 minutes before weighing. Filter solution through paper into a flask fitted with a gauze-covered cotton stopper. Sterilize by heating in an Arnold sterilizer for 1 hour before use (not essential if immediately used).

A satisfactory saline solution should not of itself be hemolytic when 1 or 2 drops of washed corpuscles are added to 5 c.c. of the solution in a test tube followed by water bath incubation for 1 hour. Neither should it be antihemolytic as

determined by the hemolysin or complement titrations. The antisheep hemolysin should give a unit of at least 0.5 c.c. of a 1:4000 dilution when titrated with 0.3 c.c. of 1:30 complement and 0.5 c.c. of a 2% suspension of washed sheep corpuscles with a water bath incubation of 1 hour. It is possible that failure of hemolysis may be due to defective hemolysin or complement or to the use of corpuscles of increased resistance to serum hemolysis, but whenever these factors may be excluded it is likely that the saline solution is defective.

If difficulties are experienced with saline prepared with distilled water, use ordinary tap water. If still unsatisfactory, add 0.1 gram of magnesium sulphate to each 1000 c.c. as recommended by Kellogg.

Preparation of Sheep Corpuscles (Indicator Antigen).—Sheep blood may be obtained at an abattoir or by bleeding a sheep from the external jugular vein (see Chapter II). In a clean (but not necessarily sterile) quart-sized Mason jar, place 30 c.c. of a 10% solution of sodium citrate in saline solution and 2 c.c. of formalin. At the abattoir have the jar almost filled with *fresh* blood (blood kept over in buckets is unsatisfactory), screw on the top, mix well with the citrate-

FIGS. 337, 338.—KOLMER
CENTRIFUGE TUBES

formalin solution and keep in a refrigerator. Ordinarily it is fit for use over a period of 2 to 3 weeks at least, but as soon as the corpuscles become too fragile a fresh supply should be secured. Boerner and Lukens advise keeping blood for 48 hours in the refrigerator before using.

If preservation is not desired, glass beads alone may be placed in the jar and after filling with blood, it should be thoroughly shaken for defibrination. Blood collected in this manner will keep at a low temperature (jar placed on a block of ice) for about a week. If beef blood is used, it may be collected by either method.

Filter a small quantity of blood through cotton into a *graduated* centrifuge tube (Figs. 337 and 338). Allow twice as much blood as the amount of cells required. Add 2 or 3 volumes of saline solution. Centrifuge at a moderate velocity until it is ascertained that all the corpuscles are thrown down.

Remove the supernatant fluid with a capillary pipet or by suction (Fig. 339). Add 3 or 4 volumes of saline solution; mix by inverting and centrifuge again for the same length of time.

Repeat the process for a third time but centrifuge twice as long as in the first washing in order to pack the cells evenly and firmly.

. Cells should be washed until the supernatant fluid is almost colorless. Three washings are usually sufficient. (If more than four washings are necessary, the cells are too fragile for use.)

Read the volume of cells in the centrifuge tube, carefully remove the supernatant fluid and prepare a 2% suspension by washing the corpuscles into a flask with 49 volumes of saline solution. *Always shake before using to secure an even suspension, as the corpuscles settle to the bottom of the flask when not in use.*

Preparation of Antisheep Hemolysin.—Give a rabbit 5 or 6 intravenous injections of 5 c.c. of a 10% suspension of washed sheep corpuscles every 5 days. Bleed the rabbit 7 to 9 days after the last injection if a preliminary titration gives a unit of 0.5 c.c. of 1:4000 or higher. Separate the serum (it need not be in-
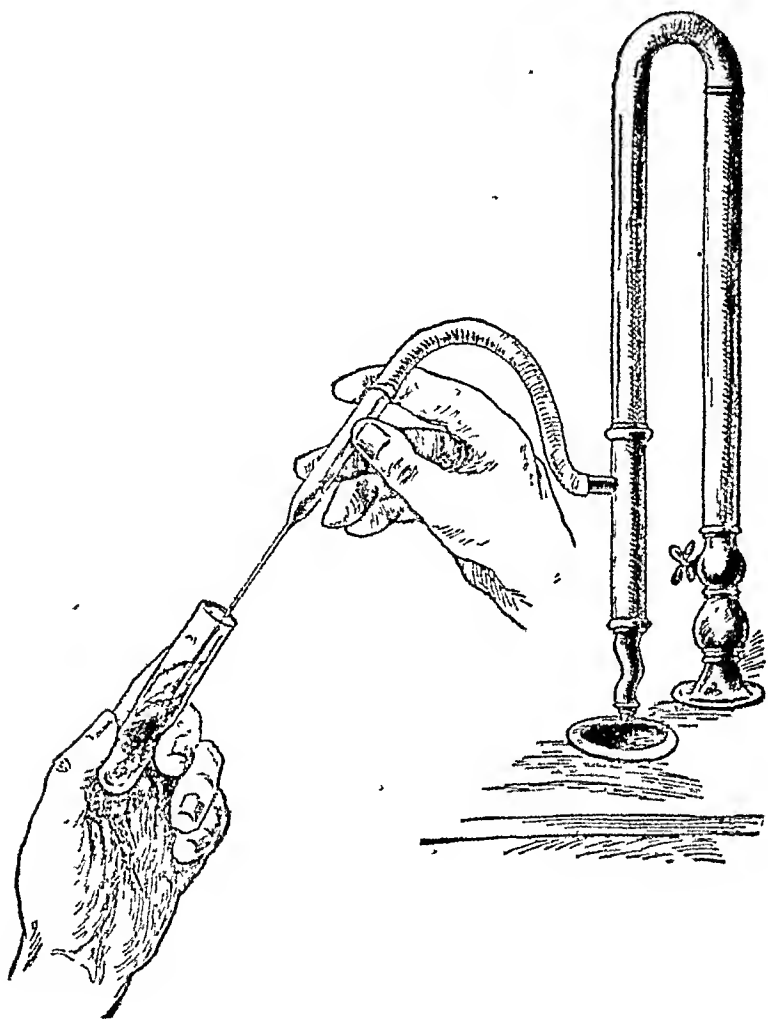
FIG. 339.—SUCTION PUMP

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

activated) and preserve with an equal part of best grade neutral glycerin. Keep in a refrigerator.

Preparation of Complement.—The pooled sera of at least 3 healthy guinea-pigs should be used. Select large, well-nourished animals that have not been fed for 12 hours; avoid pregnant animals.

Anesthetize the pig lightly with ether or stun the animal with one or two sharp blows on the head. Sever the large blood vessels on both sides of the neck, being careful not to cut the esophagus or trachea. Collect the blood in a centrifuge tube

by means of a large funnel. Place the blood in an incubator at 38° C. for 1 hour; break up the clot and centrifuge. Separate the clear serum. *Keep in the refrigerator when not in use.*

An excellent and economical method is to remove 4 to 5 c.c. of blood from the hearts of a sufficient number of large pigs (see page 42).

Complement serum may be preserved for several weeks by adding 0.3 gram of C.P. sodium chloride to each c.c. of serum. Keep in a dark glass bottle at or near the *freezing point.* To prepare for use, dilute 1 c.c. of serum with 29 c.c. of distilled water. This gives a 1:30 dilution in 1% salt solution. As preserved complement loses first in fixability by syphilis antigen and antibody, it should not be kept for more than 3 or 4 weeks. Complement may be also preserved for several weeks by adding and dissolving 1 gm. of sodium acetate (C.P.) in each 10 c.c. of serum (Rhamy). Sonnenschein's solution is also recommended (sodium acetate 12 gm.; boric acid 4 gm.; sterile distilled water 100 c.c.). Equal parts of serum and solution are mixed (1:2).

Lyophil Complement.—The most satisfactory method for preserving complement is by evaporation in the frozen state *in vacuo* by the method of Flosdorf and Mudd. (*J. Immunol.,* 1935, 29:389). It retains both hemolytic activity and fixability for 8 to 12 months and has proven satisfactory. It has the great advantage of being prepared of the pooled sera of a large number of guinea-pigs with uniform hemolytic activity and fixability by antigen and antibody. It may be obtained from Sharp and Dohme, Philadelphia, and is dispensed in vacules containing the equivalent of 5 c.c. of fresh serum. They should be kept in a refrigerator until used. By adding 5 c.c. of distilled water the material goes into immediate solution and is ready for use in the same manner as fresh serum. Preservation by the *cryochem* process is also recommended and more economical. This process has been described on page 589.

Preparation of Antigen.—A cholesterolized and lecithinized alcoholic extract of heart muscle is employed. Bacto-Beef Heart, prepared by the Digestive Ferments Company of Detroit, is recommended.

1. Place 30 grams of beef heart powder in a flask with 100 c.c. of chemically pure acetone. Stopper tightly. Keep at room temperature for 5 days with brief shaking each day.

2. Filter through fat-free paper or decant and discard the filtrate.

3. Dry the residue and extract with 100 c.c. of chemically pure absolute ethyl alcohol in a tightly stoppered flask for 5 days at room temperature, shaking each day.

4. Filter through fat-free paper with slight squeezing of the tissue.

5. Measure the filtrate and add absolute ethyl alcohol to 100 c.c.; add 0.2 gm. of cholesterol. The cholesterol is dissolved in 10 c.c. of ether and added to the alcoholic filtrate. Shake thoroughly and place in a water bath at 55° C. for 1 hour to aid solution.

6. Allow to stand at room temperature for 2 or 3 days with brief shaking each day. Filter through fat-free paper.

7. Keep the antigen at *room temperature* in a tightly stoppered bottle.

A *new antigen* of increased sensitiveness [3] with practically no change in hemolytic or anticomplementary activity may be prepared in the same manner except that it is reinforced with acetone insoluble lipoids as follows:

(*a*) Step 1, 2, 3 and 4 as above.

(*b*) Save the first 4 ether extracts used in the preparation of Kahn or Eagle antigens. Concentrate to about one-fifth volume in an evaporating dish and add 3 to 6 volumes of acetone. After mixing and setting aside overnight, the supernatant acetone is removed and the residue of acetone-insoluble lipoids kept in a refrigerator.

(*c*) Measure the alcoholic filtrate, add absolute ethyl alcohol to 100 c.c. and for each cubic centimeter add 0.002 gram of cholesterol. Dissolve the cholesterol and 1 to 2 grams of the acetone-insoluble lipoids in 20 c.c. of ether and add to the alcoholic extract in a tightly stoppered bottle or flask.

(*d*) Shake thoroughly and place in a water bath at 55° C. for 1 hour to aid in the solution of the lipoids.

(*e*) Allow to stand at room temperature for 2 or 3 days with brief shaking each day. Filter through fat-free paper.

(*f*) Keep at *room temperature*. Do not disturb any sediment that may be present.

Preparation of Sera.—1. All specimens are lined up and properly labeled.

2. The sera are removed from the clots with capillary pipets to test tubes properly labeled. Great care is required to prevent errors in labeling and confusion of sera. Each serum should be free of corpuscles, otherwise it is necessary to break up the clots with wooden applicators (one for each serum) and centrifuging for clear serum. Slight tinging with hemoglobin does no harm. Sera containing large amounts of hemoglobin are likely to be anticomplementary and unsatisfactory for both complement-fixation and precipitation tests.

3. It is not necessary to remove the natural antisheep hemolysins by absorption with *thoroughly washed sheep corpuscles* although this tends to increase the sensitiveness of complement-fixation reactions and especially in the case of sera containing small amounts of syphilis antibody. Kolmer recommends the routine removal of natural hemolysins when conditions permit in order to secure reactions of maximum sensitiveness, but when large numbers of sera require testing it may be omitted. The method for removal of natural antisheep hemolysins from sera is as follows:

(*a*) To each is added *a drop of washed sheep corpuscle sediment for approximately each 2 c.c. of blood and serum* as gaged by inspection.

(*b*) Each specimen is then thoroughly mixed with a wooden applicator (1 for each serum).

(*c*) All are then placed in a *refrigerator for 15 minutes* to enable the sheep corpuscles to absorb the hemolysins with none or but a minimum and harmless amount of hemolysis.

(*d*) All specimens are now centrifuged and the sera separated into test tubes properly labeled.

[3] J. A. Kolmer. *Am. J. Clin. Path.*, 1935, 5:55.

4. The tubes of plain or absorbed sera are now placed in a water bath at 55° C. for *15 to 20 minutes* when they are ready for testing.

Preparation of Spinal Fluids.—These are usually tested without any preliminary preparation* as they do not contain enough natural antisheep hemolysin to require removal or enough complement to require inactivation by heating at 55° C. If a specimen contains considerable blood which has not had time to settle out, it should be centrifuged. Otherwise no preparation is required as spinal fluids are tested as delivered without preliminary heating unless they are more than three days old, in which case they may be heated at 55° C. for fifteen minutes to remove thermolabile anticomplementary substances. As a general rule, however, spinal fluids are not anticomplementary unless heavily contaminated with bacteria (cloudy).

Titration of Hemolysin.—It is advisable (but not absolutely necessary) to make this titration each time the complement-fixation test is conducted.

Prepare a stock dilution of 1:100 hemolysin as follows:

Glycerinized hemolysin (50%)	2.0 c.c.
Saline solution	94.0 c.c.
Phenol (5% in saline sol.)	4.0 c.c.

This may be kept in the refrigerator for several weeks.

For titration, dilute to 1:1000 (0.5 c.c. of 1:100 + 4.5 c.c. saline solution).

In a series of 10 tubes, prepare higher dilutions as follows:

No. 1.	0.5 c.c. hemolysin (1:1000) = 1:1000
No. 2.	0.5 c.c. hemolysin (1:1000) + 0.5 c.c. saline solution = 1:2000
No. 3.	0.5 c.c. hemolysin (1:1000) + 1.0 c.c. saline solution = 1:3000
No. 4.	0.5 c.c. hemolysin (1:1000) + 1.5 c.c. saline solution = 1:4000
No. 5.	0.5 c.c. hemolysin (1:1000) + 2.0 c.c. saline solution = 1:5000
No. 6.	0.5 c.c. hemolysin (1:3000) + 0.5 c.c. saline solution = 1:6000
No. 7.	0.5 c.c. hemolysin (1:4000) + 0.5 c.c. saline solution = 1:8000
No. 8.	0.5 c.c. hemolysin (1:5000) + 0.5 c.c. saline solution = 1:10,000
No. 9.	0.5 c.c. hemolysin (1:6000) + 0.5 c.c. saline solution = 1:12,000
No. 10.	0.5 c.c. hemolysin (1:8000) + 0.5 c.c. saline solution = 1:16,000

Mix the contents of each tube thoroughly.

Prepare 1:30 dilution of complement for hemolysin and complement titrations by diluting 0.2 c.c. of complement serum with 5.8 c.c. of saline solution.

Prepare a 2% suspension of sheep corpuscles.

In a series of 10 tubes set up the hemolysin titration as shown in the table on page 623.

Mix the contents of each tube and incubate in the water bath at 37° C. for 1 hour. Read the unit of hemolysin. The *unit is the highest dilution of hemolysin that gives complete hemolysis.*

Two units are used in the complement and antigen titrations and in the comple-

ment-fixation tests. Hemolysin is so diluted that 0.5 c.c. contains 2 units. For example. if the unit equals 0.5 c.c. of 1:6000, two units equals 0.5 c.c. of 1:3000. Dilute just enough hemolysin for the complement titration and the complement-fixation tests. *Keep hemolysin and corpuscles in suspension in the refrigerator when not in use.*

HEMOLYSIN TITRATION

Tube	Hemolysin, 0.5 c.c.	Complement, c.c. (1:30)	Saline Solution. c.c.	Corpuscles, c.c.
1	1:1000	0.3	1.7	0.5
2	1:2000	0.3	1.7	0.5
3	1:3000	0.3	1.7	0.5
4	1:4000	0.3	1.7	0.5
5	1:5000	0.3	1.7	0.5
6	1:6000	0.3	1.7	0.5
7	1:8000	0.3	1.7	0.5
8	1:10,000	0.3	1.7	0.5
9	1:12,000	0.3	1.7	0.5
10	1:16,000	0.3	1.7	0.5

The following table shows how the dilutions are made so that 0.5 c.c. carries 2 units:

1 Unit 0.5 c.c. of	2 Units Would Be 0 5 c.c. of	Prepared by Diluting 1 c.c. of Stock 1:100 with Following Amounts of Saline
1:1000	1:500	4 c.c.
1:2000	1:1000	9 c.c.
1:3000	1:1500	14 c.c.
1:4000	1:2000	19 c.c.
1:5000	1:2500	24 c.c.
1:6000	1:3000	29 c.c.
1:8000	1:4000	39 c.c.
1:10,000	1:5000	49 c.c.

High titer hemolysin is recommended and the unit should be 0.5 c.c. of 1:4000 or higher.

In practice the hemolysin titration may be placed in the water bath at the same time as the complement titration; at the end of the first incubation of the complement titration the unit of hemolysin is available and 2 units added to all tubes of the complement titration, etc.

Complement Titration.—For the complement titration use 1:30 dilution of complement prepared above. *Dilute antigen so that the dose employed in the main tests is contained in 0.5 c.c.* This dilution is made by placing the required amount of saline solution in a flask and adding antigen drop by drop. Shake the flask after each addition of antigen. Prepare enough antigen dilution for the complement titration and the complement-fixation tests.

In a series of 10 test tubes set up the complement titration are as follows:

Tube	Complement, c.c. (1:30)	Antigen, Dose c.c.	Saline Solution, c.c.	Water bath 37° C. for one hour	Hemolysin, c.c. (2 units)	Corpuscles, c.c. (2 per cent)	Water bath 37° C. for one hour
1	0.1	0.5	1.4		0.5	0.5	
2	0.15	0.5	1.4		0.5	0.5	
3	0.2	0.5	1.3		0.5	0.5	
4	0.25	0.5	1.3		0.5	0.5	
5	0.3	0.5	1.2		0.5	0.5	
6	0.35	0.5	1.2		0.5	0.5	
7	0.4	0.5	1.1		0.5	0.5	
8	0.45	0.5	1.1		0.5	0.5	
9	0.5	0.5	1.0		0.5	0.5	
10	None	None	2.5		None	0.5	

The smallest amount of complement just giving complete sparkling hemolysis is the *exact unit*. The next higher tube is the *full unit* which contains 0.05 c.c. more complement. In conducting the antigen titration and complement-fixation tests, *two full units* are employed and so diluted as to be contained in 1 c.c. as per the following example:

<div style="text-align:center">

Exact unit: 0.3 c.c.

Full unit: 0.35 c.c.

Dose (two full units) : 0.7 c.c.

</div>

To calculate the dilution to employ so that 1 c.c. contains the dose of two full units, divide 30 by the dose:

$$\frac{30}{0.7} = 43 \text{ or } 1 \text{ c.c. of } 1:43 \text{ dilution of serum.}$$

The following table gives additional examples:

Exact Unit (c.c.)	Full Unit (c.c.)	Two Full Units (c.c.)	Dilution to use	Preparation
0.2	0.25	0.5	1:60	1 c.c. serum + 59 c.c. saline
0.25	0.3	0.6	1:50	1 c.c. serum + 49 c.c. saline
0.3	0.35	0.7	1:43	1 c.c. serum + 42 c.c. saline
0.35	0.4	0.8	1:37	1 c.c. serum + 36 c.c. saline
0.4	0.45	0.9	1:33	1 c.c. serum + 32 c.c. saline
0.45	0.5	1.0	1:30	1 c.c. serum + 29 c.c. saline
0.5	0.55	1.1	1:27	1 c.c. serum + 26 c.c. saline

If however the complement is unusually sensitive to the anticomplementary effects of antigen and serum in the conduct of the complement-fixation tests, as is sometimes the case during the hot summer months, 2½ exact units may be employed and so diluted that 1 c.c. contains this dose. Example:

<div style="text-align:center">

Exact unit: 0.35 c.c.

2½ units: 0.88 c.c.

</div>

To calculate the dilution to use so that 1 c.c. contains the dose, divide 30 by the dose:

$$\frac{30}{0.88} = 34 \text{ or } 1 \text{ c.c. of } 1:34 \text{ dilution of serum.}$$

The following table gives additional examples:

Unit (c.c.)	2½ Units (c.c.)	Dilution to Use	Preparation
0.2	0.5	1:60	1 c.c. serum + 59 c.c. saline
0.25	0.63	1:47	1 c.c. serum + 46 c.c. saline
0.3	0.75	1:40	1 c.c. serum + 39 c.c. saline
0.35	0.88	1:34	1 c.c. serum + 33 c.c. saline
0.4	1.0	1:30	1 c.c. serum + 29 c.c. saline
0.45	1.13	1:27	1 c.c. serum + 26 c.c. saline
0.5	1.25	1:24	1 c.c. serum + 23 c.c. saline

It is always advisable to dilute complement serum with cold saline solution instead of with saline kept at room temperature. Undiluted and especially diluted complement serum should always be kept in a refrigerator when not in use. Exposure of diluted complement to room temperature for over an hour may result in some deterioration.

Occasionally hyperactive complement yields a unit of 0.1 to 0.25 c.c. of 1:30 but when this occurs it is necessary to arbitrarily take 0.3 c.c. as the exact unit as less complement falls below the absolute minimum and is likely to be unsatisfactory.

Titration of Antigen.—It is not necessary to titrate for hemolytic and anticomplementary units as hitherto advised because titrations of over 280 antigens by Kolmer during the past 14 years have never shown any to be hemolytic in 0.5 c.c. of 1:4 and the anticomplementary units have been uniformly from 0.5 c.c. of 1:6 to not higher than 1:10. Therefore, these titrations may be omitted providing the antigen is prepared by either of the methods described.

It is, however, necessary to titrate for *antigenic activity* and either of two methods may be employed as follows:

First or Original Method.—1. Prepare a stock 1:100 dilution by adding 0.1 c.c. of antigen, drop by drop with shaking between each addition, to 9.9 c.c. of saline solution in a small flask or large test tube.

2. With this stock 1:100 dilution prepare higher dilutions as follows in test tubes:

0.1 c.c. + 0.9 c.c. saline solution = 1:1000
0.1 c.c. + 1.9 c.c. saline solution = 1:2000
0.1 c.c. + 2.9 c.c. saline solution = 1:3000
0.1 c.c. + 3.9 c.c. saline solution = 1:4000
0.1 c.c. + 4.9 c.c. saline solution = 1:5000
0.1 c.c. + 5.9 c.c. saline solution = 1:6000

0.1 c.c. + 6.9 c.c. saline solution = 1:7000

0.1 c.c. + 7.9 c.c. saline solution = 1:8000

0.1 c.c. + 8.9 c.c. saline solution = 1:9000

0.1 c.c. + 9.9 c.c. saline solution = 1:10,000

3. Prepare a 1:10 dilution of a mixture of 4 or more strongly positive syphilitic sera (like 444 — ; 442 —, etc.) heated at 55° C. for 15 to 20 minutes. Set up the titration as follows:

Tube	Antigen 0.5 c.c	Positive Serum c.c (1:10)	Complement c.c (2 full units)		Hemolysin c.c (2 units)	Corpuscles c.c (2%)	
1	1 1000	0.5	1	Shake tubes gently and place in refrigerator at 6 to 8° C. for 15 to 18 hours, followed by water bath at 37° C. for 10 minutes.	0.5	0.5	Mix thoroughly and place in water bath at 37° C. for one hour, make readings.
2	1 2000	0.5	1		0.5	0.5	
3	1 3000	0.5	1		0.5	0.5	
4	1:1000	0.5	1		0.5	0.5	
5	1:5000	0.5	1		0.5	0.5	
6	1:6000	0.5	1		0.5	0.5	
7	1:7000	0.5	1		0.5	0.5	
8	1 8000	0.5	1		0.5	0.5	
9	1:9000	0.5	1		0.5	0.5	
10	1:10,000	0.5	1		0.5	0.5	
11	Saline 0.5 c.c.	0.5	1		0.5	0.5	
12	Saline 1 c.c.	none	1		0.5	0.5	

The antigenic unit is the smallest amount of antigen producing complete (+ + + +) inhibition of hemolysis. Tube 11 is the serum control; tube 12 is the hemolytic system control. Both should show complete hemolysis. As a general rule the unit is 0.5 c.c. of 1:2000 to 1:4000 dilutions with a higher unit in the case of antigen reënforced with acetone-insoluble lipoids.

In the complement-fixation tests 10 antigenic units of antigen are used. The antigen is so diluted that 0.5 c.c. contains the dose. For example:

Antigenic unit = 0.5 c.c. of 1:3000
Dose (10 units) = 0.5 c.c. of 1:300

If, however, the unit is 0.5 c.c. of 1:5000 or higher, as is frequently the case with antigen reënforced with acetone-insoluble lipoids, it may be used in dose of 20 units. For example:

Antigenic unit = 0.5 c.c. of 1:6000
Dose (20 units) = 0.5 c.c. of 1:300

As a general rule this dose of 10 or 20 units is 30 to 60 times less than the anticomplementary unit, which largely accounts for the high specificity of the reaction.

Both antigens will keep for a year or longer at room temperature with no change in antigenic or anticomplementary properties.

Second Method—As recommended by Boerner and Lukens.[4] It has the advantage of giving approximately the same dose to employ in the main test regardless of the amount of complement fixing antibody in the serum used in conducting the titration. Furthermore, it is a check on prezone reactions and shows the optimum dose to employ in conducting diagnostic tests.

1. Prepare a 1:80 dilution of antigen by adding 0.1 c.c. drop by drop with shaking between each, to 7.9 c.c. of saline solution in a large test tube or small flask. Higher dilutions are then prepared as follows:

$$4\ \text{c.c. of } 1:80\ +\ 4\ \text{c.c. saline solution} = 1:160$$
$$4\ \text{c.c. of } 1:160\ +\ 4\ \text{c.c. saline solution} = 1:320$$
$$4\ \text{c.c. of } 1:320\ +\ 4\ \text{c.c. saline solution} = 1:640$$
$$4\ \text{c.c. of } 1:640\ +\ 4\ \text{c.c. saline solution} = 1:1280$$
$$4\ \text{c.c. of } 1:1280\ +\ 4\ \text{c.c. saline solution} = 1:2560$$

2. Arrange 5 rows of test tubes with 6 in each row. In the *first* tube of each row, place 0.5 c.c. of antigen 1:80; to the *second* tube of each row, 0.5 c.c. of antigen 1:160; to the *third* tube, 0.5 c.c. of 1:320; to the *fourth*, 0.5 c.c. of 1:640; to the *fifth* 0.5 c.c. of 1:1280 and to the *sixth* 0.5 c.c. of 1:2560.

3. Heat 3 c.c. of a moderately to strongly positive syphilitic serum in a water bath at 55° C. for 15 to 20 minutes and prepare 5 dilutions as follows in large test tubes:

1.0 c.c. serum + 4.0 c.c. saline = 1:5   (0.5 c.c. carries 0.1   c.c. serum)
0.5 c.c. serum + 4.5 c.c. saline = 1:10 (0.5 c.c. carries 0.05   c.c. serum)
0.5 c.c. serum + 9.5 c.c. saline = 1:20 (0.5 c.c. carries 0.025 c.c. serum)
2.0 c.c. serum 1:20 + 2.0 c.c. saline = 1:40   (0.5 c.c. carries 0.0125 c.c. serum)
1.0 c.c. serum 1:20 + 4.0 c.c. saline = 1:100 (0.5 c.c. carries 0.005   c.c. serum)

4. Add 0.5 c.c. of 1:5 dilution to each of the 6 tubes of the first row; 0.5 c.c. of 1:10 to each tube of the second row; 0.5 c.c. of 1:20 to each tube of the third row; 0.5 c.c. of 1:40 to each tube of the fourth row and 0.5 c.c. of 1:100 to each tube of the fifth row.

5. Add 1 c.c. of complement dilution carrying 2 full units to all tubes.

6. Put up a *serum control* carrying 0.5 c.c. of 1:5 serum and 1 c.c. of complement (2½ units); also a *hemolytic system control* carrying 1 c.c. of saline solution and 1 c.c. of complement (2½ units).

7. Shake the tubes gently and place in refrigerator at 6° to 8° C. for 15 to 18 hours, followed by water bath at 37° C. for 10 minutes.

8. Add 0.5 c.c. of hemolysin (2 units) and 0.5 c.c. of 2% suspension of corpuscles to all tubes.

9. Mix thoroughly and place in a water bath at 37° C. for one hour; make readings. The serum and hemolytic system controls should show complete hemolysis.

[4] Boerner, F., and Lukens, M.: *Am. J. Clin. Path.*, 1937, 7, 33. Kolmer, J. A.: *Am. Jour. Clin. Path.*, 1937, 7, 155. The principles of this method were previously described by Hooker, S. B.: *Jour. Immunology*, 1927, 14, 129.

10. Chart the results as per the following example observed with a strongly positive serum:

Serum in 0.5 c.c.	Antigen in 0.5 c.c. Amounts					
	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280	1 : 2560
0.005	–	–	++	–	–	+
0.0125	–	+	++++	++	++	+
0.025	+	++++	++++	++++	–	+
0.05	++++	++++	++++	++++	++++	++
0.1	++++	++++	++++	++++	++++	+++

11. The dose of antigen to employ in the main tests is the *largest amount giving a + + + + reaction with the smallest amount of serum*. If three dilutions of antigen give + + + + reactions with the smallest amount of serum, the dose to use should be midway between the highest and lowest.

In the above example the dose would be 0.5 c.c. of 1:320. The results of a number of titrations by this method compared with the first method have shown that a dose of 10 or 20 units by the latter is usually closely similar to the dose determined by the above method. Kolmer believes, therefore, that either method may be employed and approves this second method because of the correctness of the principles involved, since it determines the correct dilution to use in dose of 0.5 c.c. regardless of whether the titration is conducted with strongly, moderately or weakly positive serum.

Choice of Complement-Fixation Methods.—Two methods are available. One, the *quantitative test*, employs 5 doses of serum [a] or spinal fluid and is generally preferred, especially in testing the sera of cases of syphilis under treatment in order to secure serological evidences of improvement. The second, designated as the *qualitative test*, uses 2 doses of serum 0.2 and 0.1 c.c. with an additional third tube as a control (0.2 c.c.) and is sufficient for diagnostic purposes as well as being more economical of materials and time required. Both are of equal sensitiveness and specificity.

The Quantitative Complement-Fixation Test.—1. For each serum: (a) Arrange 6 test tubes and place the following amounts of saline solution respectively: 0.9, 0.5, 0.5, 0.5, 2.0 and 0.5 c.c.

(b) To tube No. 1 add 0.6 c.c. of inactivated serum. Mix by drawing up in the pipet several times and transfer 0.5 c.c. to No. 2 and 0.5 c.c. to No. 6 (serum control).

(c) Mix No. 2 and transfer 0.5 c.c. to No. 3.

(d) Mix No. 3 and transfer 0.5 c.c. to No. 4.

(e) Mix No. 4 and transfer 0.5 c.c. to No. 5; mix and discard 2.0 c.c.

This leaves 0.5 c.c. in each of the first 5 tubes carrying the following amounts of serum 0.2, 0.1, 0.05, 0.025 and 0.005 c.c.; No. 6 (the serum control) carries 1.0 c.c. or 0.2 c.c. of serum since it receives no antigen and thereby making the total volume in all tubes the same when the test is finished.

[a] Changed to 0.2, 0.1, 0.05, 0.025 and 0.005 c.c. with 0.2 c.c. in the serum control as suggested by Boerner and Lukens, *Am. J. Syph.*, 1935, 19:489.

QUANTITATIVE REACTION.

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

10. Chart the results as per the following example observed with a strongly positive serum:

Serum in 0.5 c.c.	Antigen in 0.5 c.c. Amounts					
	1:80	1:160	1:320	1:640	1:1280	1:2560
0.005	−	−	++	−	−	−
0.0125	−	+	+++	+++	++	+
0.025	+	++++	++++	++++	+++	+
0.05	++++	++++	++++	++++	++++	++
0.1	++++	++++	++++	++++	++++	+++

11. The dose of antigen to employ in the main tests is the *largest amount giving a + + + + reaction with the smallest amount of serum.* If three dilutions of antigen give + + + + reactions with the smallest amount of serum, the dose to use should be midway between the highest and lowest.

In the above example the dose would be 0.5 c.c. of 1:320. The results of a number of titrations by this method compared with the first method have shown that a dose of 10 or 20 units by the latter is usually closely similar to the dose determined by the above method. Kolmer believes, therefore, that either method may be employed and approves this second method because of the correctness of the principles involved, since it determines the correct dilution to use in dose of 0.5 c.c. regardless of whether the titration is conducted with strongly, moderately or weakly positive serum.

Choice of Complement-Fixation Methods.—Two methods are available. One, the *quantitative test*, employs 5 doses of serum[a] or spinal fluid and is generally preferred, especially in testing the sera of cases of syphilis under treatment in order to secure serological evidences of improvement. The second, designated as the *qualitative test*, uses 2 doses of serum 0.2 and 0.1 c.c. with an additional third tube as a control (0.2 c.c.) and is sufficient for diagnostic purposes as well as being more economical of materials and time required. Both are of equal sensitiveness and specificity.

The Quantitative Complement-Fixation Test.—1. *For each serum:* (a) Arrange 6 test tubes and place the following amounts of saline solution respectively: 0.9, 0.5, 0.5, 0.5, 2.0 and 0.5 c.c.

(b) To tube No. 1 add 0.6 c.c. of inactivated serum. Mix by drawing up in the pipet several times and transfer 0.5 c.c. to No. 2 and 0.5 c.c. to No. 6 (serum control).

(c) Mix No. 2 and transfer 0.5 c.c. to No. 3.

(d) Mix No. 3 and transfer 0.5 c.c. to No. 4.

(e) Mix No. 4 and transfer 0.5 c.c. to No. 5; mix and discard 2.0 c.c.

This leaves 0.5 c.c. in each of the first 5 tubes carrying the following amounts of serum 0.2, 0.1, 0.05, 0.025 and 0.005 c.c.; No. 6 (the serum control) carries 1.0 c.c. or 0.2 c.c. of serum since it receives no antigen and thereby making the total volume in all tubes the same when the test is finished.

[a] Changed to 0.2, 0.1, 0.05, 0.025 and 0.005 c.c. with 0.2 c.c. in the serum control as suggested by Boerner and Lukens, *Am. J. Syph.*, 1935, 19:489.

S. C.   0.005   0.025   0.05   0.1   0.2

QUANTITATIVE REACTION.

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

2. *For each spinal fluid:*

(*a*) Arrange six tubes and place 0.5 c.c. saline solution in tubes 2. 3, 4, 5 and 6.

(*b*) In tubes 1, 2 and 6 place 0.5 c.c. spinal fluid. Mix No. 2 and transfer 0.5 c.c. to No. 3. Mix No. 3 and transfer 0.5 c.c. to No. 4. Mix No. 4 and transfer 0.5 c.c. to No. 5. Mix No. 5 and discard 0.5 c.c.

(*c*) Tubes 1 to 5 now contain 0.5 c.c. carrying 0.5, 0.25, 0.125, 0.0625 and 0.03125 c.c. of spinal fluid. Tube 6 (control) contains 1 c.c. carrying 0.5 c.c. of spinal fluid.

3. To the first 5 tubes of each set of serum or spinal fluid add 0.5 c.c. of diluted antigen carrying the proper dose.

4. After an interval of 10 to 30 minutes, add 1 c.c. complement (2 full units) to each tube.

5. Include the following controls:

Antigen control containing 0.5 c.c. diluted antigen, 0.5 c.c. saline solution and 1.0 c.c. diluted complement (2 full units).

Hemolytic system control containing 1 c.c. saline solution and 1 c.c. diluted complement.

Corpuscle control containing 2.5 c.c. salt solution.

Positive and negative serum controls should be included.

6. Mix the contents of each tube by gently shaking and place in the refrigerator at 6° to 8° C. for 15 to 18 hours.

7. Place tubes in the water bath at 37° C. for 10 to 15 minutes (not longer).

8. To all tubes, except the corpuscle control, add 0.5 c.c. of hemolysin (carrying 2 units) and to all tubes add 0.5 c.c. of 2% corpuscle suspension (shaken up).

9. Mix the contents of each tube by gently shaking and place in the water bath at 37° C. for 1 hour when the readings are made (Plate XI). *More sensitive readings may be made 10 minutes after the antigen, hemolytic system and serum controls show complete hemolysis and is preferred.*

10. The following table shows the set-up for the quantitative complement-fixation test with serum:

Tube	Patient's Serum in 0.5 c c	Antigen, c c		Complement, c c (2 full units)		Hemolysin, c c (2 units)	Corpuscles, c.c (2 per cent)	
1	0.2 c.c.	0.5		1.0		0.5	0.5	
2	0.1 c.c.	0.5		1.0		0.5	0.5	
3	0.05 c.c.	0.5		1.0		0.5	0.5	
4	0.025 c.c.	0.5	Interval of 10 to 30 minutes at room temperature	1.0	Primary incubation in refrigerator at 6° to 8° C. for 15 to 18 hours, followed by 10 to 15 minutes at 37° C.	0 5	0.5	Secondary incubation in water at 37° C.
5	0.005 c.c.	0.5		1.0		0.5	0.5	
6	0.2 c.c. (control)	None		1.0		0.5	0.5	
7	Antigen control: 0.5 c.c. saline solution	0.5		1.0		0.5	0 5	
8	Hemolytic control· 1.0 c.c. saline solution	None		1.0		0.5	0.5	
9	Corpuscle control: 2.5 c.c. saline solution	None		None		None	0.5	

11. After the secondary incubation the readings may be made at once or after the tubes have been placed in the refrigerator for several hours to permit the settling of nonhemolyzed corpuscles. Read the degree of inhibition of hemolysis and record for each tube as: — (complete hemolysis); + (25% inhibition recorded as 1); ++ (50% inhibition recorded as 2); +++ (75% inhibition recorded as 3); ++++ (100% inhibition recorded as 4). All serum, antigen and hemolytic controls should show complete hemolysis. The corpuscle control should show no hemolysis.

12. Reactions may be interpreted as follows: (See Plate XI):

(a) *Very strongly positive* when *complete fixation* (++++) occurs in the third and fourth or fifth tubes. Examples: 44444; 4444—; 4442—; 444——; 344——.

(b) *Strongly positive* when *complete fixation* (++++) occurs in the second tube. Examples: 4431—; 442——; 342——; 44————.

(c) *Moderately positive* when *complete* fixation (++++) occurs in first tube only. Examples: 431——; 42———; 4————.

(d) *Weakly positive* when *partial* fixation occurs in one or more tubes. Examples: 321——; 21————; 1————.

(e) *Doubtfully positive* when the reaction is ± in the first tube. Example: ±————.

(f) *Negative* when there is complete hemolysis in all tubes. Example: —————.

The method of recording and reporting a complement-fixation test by this method is according to the following examples:

Quantitative reaction	= strongly positive (442——)
Serum 0.2 c.c.	= ++++
Serum 0.1 c.c.	= ++++
Serum 0.05 c.c.	= ++
Serum 0.025 c.c.	= ~
Serum 0.005 c.c.	= ~
Serum 0.2 c.c. (control)	= —

Quantitative reaction	= strongly positive (42——⌐)
Spinal fluid 0.5 c.c.	= ++++
Spinal fluid 0.25 c.c.	= ++
Spinal fluid 0.125 c.c.	= —
Spinal fluid 0.0625 c.c.	= —
Spinal fluid 0.03125 c.c.	= —
Spinal fluid 0.5 (control)	= —

The American Committee on Evaluation of Serodiagnostic Tests for Syphilis has recommended reporting reactions only as positive, doubtful or negative.

The Qualitative Complement-Fixation Test.—1. This test is conducted in exactly the same manner as described for the quantitative test except that 3 doses of serum (0.2 and 0.1 c.c. with 0.2 c.c. in the serum control) are employed. With spinal fluid a single dose of 0.5 c.c. with 0.5 c.c. in the control is employed.

2. For each *serum* arrange 3 test tubes and place the following amounts of saline solution: 0.9, 0.5 and 0.5 c.c.

To No. 1 add 0.6 c.c. of inactivated serum. Mix by drawing up in the pipét several times and transfer 0.5 c.c. to No. 2 and 0.5 c.c. to No. 3 (serum control). Mix No. 2 and discard 0.5 c.c.

This leaves 0.5 c.c. in each of the first 2 tubes carrying 0.2 and 0.1 c.c. of serum respectively. No. 3 contains 1.0 c.c. (0.2 c.c. of serum) since it receives no antigen and thereby making the total volume in all tubes the same when the test is finished.

3. For each *spinal fluid* arrange 2 tubes and place 0.5 c.c. in each. The first tube receives antigen; the second does not and is the control to which add 0.5 c.c. saline solution.

4. Place 0.5 c.c. of proper dilution of antigen in Nos. 1 and 2 of each serum and in No. 1 of each spinal fluid; also in control tube carrying 0.5 c.c. saline (antigen control).

5. Allow tubes to stand 10 to 30 minutes. Then add 2 full units of complement (1.0 c.c.) to each; also to a control carrying 1 c.c. saline (hemolytic system control).

6. Put up corpuscle control: 2.5 c.c. saline and 0.5 c.c. of 2% suspension.

7. Mix tubes gently and place in refrigerator at 6° to 8° C. for 15 to 18 hours. Keep hemolysin and corpuscle suspension in refrigerator.

8. Place tubes in water bath at 37° C. for 10 to 15 minutes (not longer).

9. To all tubes except corpuscle control add 2 units of hemolysin.

10. To all tubes except corpuscle control add 0.5 c.c. of 2% corpuscle suspension (shaken up).

11. Mix and place in water bath for 1 hour when the readings are made. Or place the tubes in a refrigerator for an hour or two before making the readings. *More sensitive readings may be made 10 minutes after the antigen, hemolytic systems and serum controls show complete hemolysis and is preferred.*

12. The serum, hemolytic system and antigen controls should be completely hemolyzed; the corpuscle control should show no hemolysis.

13. Read and record the *serum* tests as follows:

(*a*) *Strongly positive:* complete fixation ($+ + + +$) in second tube. Examples: 44; 34.

(*b*) *Moderately positive:* Complete fixation ($+ + + +$) in first tube only. Examples: 42; 41.

(*c*) *Weakly positive:* Partial fixation in one or both tubes. Examples: 31; 21; 3—; 2—; 1—.

(*d*) *Doubtfully positive:* ± in first tube. Example: ± —

(*e*) *Negative:* Complete hemolysis in both tubes.

14. Read and record the *spinal fluid* tests as follows according to first tube:

$$+ + + + = Strongly\ positive$$
$$+ + + = Moderately\ positive$$
$$+ + \text{ or } + = Weakly\ positive$$
$$\pm = Doubtfully\ positive$$
$$- = Negative$$

The American Committee on Evaluation of Serodiagnostic Tests for Syphilis has recommended reporting reactions only as positive, doubtful or negative.

Modified Technic for Small Amounts of Serum and Spinal Fluid.—It sometimes occurs that a sufficient amount of serum or spinal fluid is not available for conducting the quantitative test. In such cases the test can be conducted in exactly the same manner as described *using all reagents in one-half the usual amounts.* The hemolysin and complement are not titrated separately for this test as the same dilutions are employed in one-half amounts. The same antigen dilution is also used but in one-half amount.

1. For each *serum:*

(*a*) Arrange 6 test tubes and place the following amounts of saline respectively: 1.2, 0.5, 0.5, 0.5, 2.0 and 0.25 c.c.

(*b*) To tube No. 1 add 0.3 c.c. of inactivated serum. Mix by drawing up in the pipet several times and transfer 0.5 c.c. to No. 2 and 0.5 c.c. to No. 6 (serum control).

(*c*) Mix No. 2 and transfer 0.5 c.c. to No. 3.

(*d*) Mix No. 3 and transfer 0.5 c.c. to No. 4.

(*e*) Mix No. 4 and transfer 0.5 c.c. to No 5; mix and discard 2.0 c.c.

This leaves 0.5 c.c. in each of the 5 tubes carrying exactly ½ the amounts of serum recommended for the regular test, namely: 0.1, 0.05, 0.025, 0.0125 and 0.0025 c.c.; No. 6 (serum control) carries 0.1 c.c. of serum.

2. For each *spinal fluid:*

(*a*) Arrange 6 test tubes and place the following amount of saline respectively: 0.75, 0.5, 0.5, 0.5, 0.5 and 0.25 c.c.

(*b*) To tube No. 1 add 0.75 c.c. of spinal fluid. Mix and transfer 0.5 c.c. to tube No. 2 and 0.5 c.c. to tube No. 6.

(*c*) Mix No. 2 and transfer 0.5 c.c. to No. 3.

(*d*) Mix No. 3 and transfer 0.5 c.c. to No. 4.

(*e*) Mix No. 4 and transfer 0.5 c.c. to No. 5.

(*f*) Mix No. 5 and discard 0.5 c.c.

This leaves 0.5 c.c. in each of the 5 tubes carrying exactly ½ the amount of spinal fluid recommended in the regular test, namely: 0.25, 0.125, 0.0625, 0.0312 and 0.0156 c.c.

3. Proceed as directed on page 628 for the quantitative test, using exactly ½ the amount of each reagent. Start with step 3.

4. Readings are reported in the same manner as described for the regular test.

5. A *qualitative* test may be conducted by placing 0.1, 0.05 and 0.1 c.c. (control) of serum in each of three test tubes. With spinal fluid place 0.25 c.c. in each of two tubes (the second being the control). Add sufficient saline to make the total volume 0.5 c.c. in each tube of the test with 0.75 c.c. in the control.

6. Proceed as directed on page 630 for the qualitative test, using exactly ½ the amount of each reagent. Start with step 4.

7. Readings are reported in the same manner as described for the regular test.

Analysis of Difficulties.—*Defective Complement.*—In the great majority of instances difficulties are due to complement supersensitive to the anticomplementary effects of antigen, serum or both. This is especially likely to occur during the hot months of the year. Sometimes complement may be defective in hemolytic activity and whenever the unit is higher than 0.5 c.c. of 1:30 dilution it should not be used. But sometimes the complement is satisfactory from this standpoint but yet defective in the tests because supersensitive to antigen and prone to give prezone reactions. With negative or normal sera the reactions are apt to be — 1 2 — — — or — 1 2 4 4 with perfect serum controls. Since 2½ units of complement have been used instead of 2 full units as originally advised, this difficulty has been greatly reduced. Furthermore, under these conditions lyophil or cryochem complement is recommended since the complement is a mixture of the sera of a large number of guinea-pigs and may be prepared during the colder months of the year. When fresh serum is used it is important to prepare it from several *full grown,* healthy and *previously* unused pigs. As a general rule the trouble is first thought to be due to defective hemolysin but, since this reagent keeps very well, it is seldom responsible.

Defective Saline Solution.—When trouble is experienced with the hemolytic system when first using these methods, it is likely that the saline solution is at fault. If it has been prepared with distilled water, try a saline prepared by dissolving 8.5 grams of chemically pure sodium chloride in 1000 c.c. of ordinary tap water; it is sometimes advantageous to add 0.1 gram of magnesium sulphate. If, however, trouble is experienced after a saline has been previously used with success it is unlikely to be the cause. Compressed tablets of salt should not be used.

Defective Hemolysin.—This is probably least likely to be a cause of trouble although usually first suspected, especially if the hemolysin has been previously used with success. The unit of antisheep hemolysin should be at least 0.5 c.c. of 1:4000 and sera of this and higher strengths are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory, a good hemolysin is rarely responsible even when shipped over long distances.

Defective Corpuscles.—When blood is obtained from an abattoir one is almost sure sooner or later to encounter the corpuscles of occasional animals possessing *increased resistance* to serum hemolysis. The cause of this phenomenon is unknown; fortunately it is rare. The remedy is to discard the corpuscles and secure a fresh supply of blood.

Anticomplementary Antigen.—Providing no mistakes have occurred in dilution and dosage, this is very rarely a cause of trouble. When the antigen control shows incomplete hemolysis it is almost surely due to some component of the hemolytic system, especially the complement.

Anticomplementary Sera.—Sera and spinal fluids may be found to be anticomplementary, as shown by incomplete hemolysis of the serum controls. After experience has been gained some of these reactions may be safely read, but as a general rule, it is safer and wiser to repeat the tests with fresh serum, especially in the case of those technicians lacking experience in complement-fixation work. It is

infinitely better to repeat the tests than to run the slightest chances of error, especially the regrettable and almost unpardonable error of rendering a falsely positive report. Sometimes the majority of sera of a day's work show incomplete hemolysis of the serum controls, but this trouble is not due to anticomplementary effects on their part but rather to the use of a defective supersensitive complement. Under these conditions the tests must be repeated and for this reason *the unused portions of all sera should be routinely kept in a refrigerator until the tests are completed in case repetitions are required.*

COMPLEMENT-FIXATION TESTS WITH ANTICOMPLEMENTARY SERA, SPINAL FLUIDS, EXUDATES AND TRANSUDATES

As is well known sera, spinal fluids, exudates and transudates (chancre secretions, pleural, pericardial, and peritoneal exudates and transudates) may become anticomplementary or capable in themselves of fixing or inactivating complement in the *absence* of antigen; this is especially true of old and contaminated sera, chancre and pleural exudates, by reason of the presence of bacteria. In routine complement-fixation tests the phenomenon is detected by the occurrence of incomplete hemolysis in the serum controls. Since any serum, spinal fluid, etc., may contain anticomplementary substances, one should never assume that they are absent but always and invariably include the serum controls in every complement-fixation test.

First Method.—It is possible to read safely some anticomplementary reactions *when the serum controls show only slight interference with hemolysis.* The following are examples in which *negative* reports were correctly rendered:

```
1————1
2————1
2 1————2
2————2
4————2
4————3
```

In other words, if the first tube shows the same or very nearly the same degree of inhibition of hemolysis as the serum control with none or but the slightest inhibition in the second tube it is practically certain that the serum contains no antibody and that a negative report may be rendered; indeed, the evidence of a negative reaction is even stronger under these conditions than if the serum were free of anticomplementary action because if even a trace of antibody were present, strong inhibition of hemolysis would occur in the second and even the third tubes of the quantitative test.

Positive reports are also possible of correct reading, especially by *experienced* serologists *whenever the serum control shows less than* $+++$ (3) reactions, although it is impossible to estimate the degree of positiveness (one reporting only a "positive" reaction):

$$4 \ 4 \ 3 \ — \ —2$$
$$3 \ 2 \ — \ — \ —1$$
$$4 \ 1 \ — \ — \ —1$$
$$4 \ 4 \ 4 \ 4 \ —2$$

It is true, however, that some risk of error may accompany reports of this kind and it is always advisable to repeat the tests; this is especially true in the case of inexperienced workers.

Whenever the serum control shows complete inhibition of hemolysis ($+ + + +$ or 4), it is too risky to report, as, for example, in the following reactions:

$$4 \ 4 \ 1 \ — \ —4$$
$$4 \ 2 \ — \ — \ —4$$
$$4 \ 4 \ 3 \ — \ —4$$

Second Method.—Sera *deeply* stained with hemoglobin are always likely to be anticomplementary; likewise serum, spinal fluid, urine, milk, etc., containing large numbers of bacteria, and freshly collected chancre exudates.

When these are tested it is advisable to put up a control on each dose; this is readily accomplished in routine work by setting up two series of 5 tubes in the usual manner; antigen is added to all tubes of the first row but not to the second row, which serves as the controls on each dose of serum or spinal fluid. The following are examples of reaction employing the quantitative test:

First row: 4 4 4 1 —
Second row: 4 1 — — — } Syphilitic serum

First row: 4 4 4 1 —
Second row: 4 1 — — — } Syphilitic serum

First row: 4 4 1 — —
Second row: 3 — — — — } Syphilitic spinal fluid

First row: 4 2 — — —
Second row: 4 1 — — — } Nonsyphilitic serum

First row: 3 2 — — —
Second row: 3 1 — — — } Nonsyphilitic serum

First row: 4 4 2 — —
Second row: 4 3 1 — — } Nonsyphilitic spinal fluid

Whenever complement fixation in the first row is *markedly* greater than in the second row, as shown above with the two syphilitic sera and spinal fluid, one may safely report a positive reaction without attempting to express an opinion on the degree of positiveness; negative reactions may be reported when the differences between the first and second rows are very slight. But whenever the differences are less marked, it is unsafe and unwise to vouchsafe a positive report as, for example, in reactions of this kind conducted with known sera allowed to become anti-complementary:

| First row: | 4 | 4 | 4 | 2 | — | } Syphilitic serum |
| Second row: | 4 | 4 | 1 | — | — | |

| First row: | 3 | 1 | — | — | — | } Syphilitic serum |
| Second row: | 2 | — | — | — | — | |

| First row: | 2 | 1 | — | — | — | } Syphilitic serum |
| Second row: | 1 | — | — | — | — | |

| First row: | 4 | 4 | 4 | 2 | 1 | } Syphilitic serum |
| Second row: | 4 | 4 | 4 | 1 | — | |

| First row: | 4 | 3 | 2 | — | — | } Syphilitic spinal fluid |
| Second row: | 4 | 2 | — | — | — | |

It is true that the first row carrying antigen almost always shows stronger reactions than the second row, but unless the differences are quite marked it is not safe to give a positive report because normal or negative but anticomplementary serum and spinal fluid always yield a greater degree of inhibition of hemolysis in the presence of antigen.

Modified Sachs' Method for Anticomplementary Sera.—Sachs has described a very useful method for testing anticomplementary sera. Sera very deeply tinged with hemoglobin do not respond quite as well to this method. Rabbit and dog sera, and the sera of other of the lower animals may be treated in the same manner since it likewise removes the anticomplementary substances from the majority but not the substances responsible for the nonspecific complementary-fixation reactions sometimes yielded by the normal sera of rabbits, dogs, and mules. The method has not been applied to spinal fluids.

1. Heat 0.5 c.c. of serum at 55° C. for 15 minutes.

2. Add 4.1 c.c. of accurately titrated N/300 hydrochloric acid and mix.

3. After standing ½ hour at room temperature, centrifuge thoroughly and discard the sediment.

4. To the supernatant fluid add 0.4 c.c. of 10% sodium chloride solution. The acid is fixed by the precipitate of globulin; hence neutralization is unnecessary.

5. This gives a 1:10 dilution of original serum ready for testing.

6. Arrange two rows of 5 test tubes (the rear row are serum controls and receive no antigen).

7. Place 1 c.c. of normal saline solution in tubes 3 and 4 and 2 c.c. in tube 5 of the first row; place 0.5 c.c. in each of the 5 tubes of the second row.

8. Place 1 c.c. of serum diluted 1:10 in first and third tubes of the first row and 0.5 c.c. in the second tube. Mix No. 3 and transfer 1 c.c. to No. 4 and 0.5 c.c. to No. 3 of the second row. Mix No. 4; transfer 0.5 c.c. to No. 5, 0.5 c.c. to No. 4 of second row and discard 0.5 c.c. Mix No. 5, transfer 0.5 c.c. to No. 5 of the rear row and discard 1.5 c.c.

9. Place 1 c.c. of serum diluted 1:10 in No. 1 and 0.5 c.c. in No. 2 of the second row.

10. Add antigen (0.5 c.c. of proper dilution as used in the regular test) to each tube of the front row. Allow to stand at room temperature for 10 to 30 minutes

when 2 full units of complement are added to all tubes of both rows and the balance of the test completed in the usual manner.

11. The tubes of the front and rear rows carry 0.1, 0.05. 0.025, 0.0125 and 0.0025 c.c. of serum respectively.

12. Upon completion of the test all of the tubes of the second row should show complete hemolysis. However, the first tube carrying 0.1 c.c. and sometimes the second carrying 0.05 c.c. of serum, may not show complete hemolysis. With negative sera the corresponding front tubes show the same degree of inhibition of hemolysis and under these conditions a negative report may be rendered. With positive sera inhibition of hemolysis is much more marked in the tubes of the front row. It is advisable to report the reactions as positive, doubtful or negative.

KOLMER COMPLEMENT-FIXATION TESTS FOR TUBERCULOSIS, GONORRHEA, TYPHOID FEVER, GLANDERS, CONTAGIOUS ABORTION AND OTHER BACTERIAL DISEASES

The technic of the Kolmer bacterial complement-fixation tests is the same as for his syphilis test. The methods of preparing the antigens are of most importance.

The *hemolytic system* is exactly the same as described and the *hemolysin* is titrated in the same manner. Likewise the *complement,* which is titrated in the presence of the antigen employed in the dose of the main tests. The amounts of *serum* employed in the quantitative and qualitative tests are the same as in the syphilis test. The *primary incubation* is the same except that after incubation at 6° to 8° C. for 15 to 18 hours. water bath incubation at 37° C. is for 30 minutes instead of the 10 to 15 minutes recommended for the syphilis test. A substitute primary incubation of 2 hours at 37° C. in a water bath may be used, especially in conducting the tuberculosis complement-fixation test.

Preparation of Bacterial Antigens.—*Tuberculosis.*—1. Cultivate human tubercle bacilli in glycerin broth for about 4 weeks and autoclave the flasks at 10 pounds pressure for 20 minutes to kill the organisms.

2. Filter on several layers of good paper and wash the bacillary residue free of glycerin with sterile water.

3. Transfer the residue to a desiccator and dry over sulphuric acid.

4. Grind in a mortar under a hood for ½ hour and keep in a tightly stoppered bottle at room temperature.

5. Place 1 gram of powder in a small Erlenmeyer flask fitted with a Liebig's condenser and electric heater and boil gently for 1 hour with 200 c.c. of ether. Discard the ether: dry the residue by placing the flask in an incubator, add 200 c.c. of acetone and boil for 1 hour. Discard the acetone, add 200 c.c. of absolute ethyl alcohol and boil for 1 hour. Discard the alcohol.

6. Dry the residue in the flask, add 190 c.c. of distilled water and boil for 1 hour. Add 2 grams sodium chloride to render isotonic and 10 c.c. of 5% tricresol or phenol as a preservative. Store in a tightly stoppered bottle in a refrigerator for at least one week to ripen before titration.

Gonococci, Typhoid Bacilli, Glanders Bacilli, Brucella abortus, Streptococci and Other Organisms.—FIRST METHOD.—This method is especially adapted for preparing antigens of the gonococcus and other organisms difficult of cultivation on a large scale. Both it and the method that follows are based upon the principle of utilizing whole organisms. In the preparation of gonococcus antigen it is important to use several strains which represent the several different serologic types (Torrey).

1. Cultivate the organism on a suitable solid medium and wash off the growths with sufficient sterile distilled water to give a suspension containing approximately 2,000,000,000 per c.c. Or the organism may be cultivated in a suitable fluid medium, centrifugated and the residue suspended in sterile water to the same concentration.

2. Shake the suspension with glass beads for an hour to break up clumps.

3. Transfer to an Erlenmeyer flask fitted with a Liebig's condenser and electric heater and boil gently for 2 hours. Or the suspension may be boiled in an Arnold sterilizer for the same time, making up for any loss in volume with sterile water.

4. Add 1 gram of sodium chloride and 5 c.c. of 5% tricresol or phenol to each 100 c.c.; stopper tightly and keep in a refrigerator for a week to ripen before titrating.

Second Method.—This method is particularly serviceable for preparing antigens of organisms readily secured in large amounts, as storing in dry powdered form provides a means for keeping indefinitely the base from which antigen may be made up in small amounts as required.

1. Cultivate the organism on a suitable solid medium and remove with a *minimum* amount of sterile saline solution or cultivate in a fluid medium and secure the organisms by centrifugation.

2. Dry the residue in a desiccator over sulphuric acid and grind for ½ hour under a hood.

3. Store the powder in ampules or in a tightly stoppered bottle at room temperature.

4. For use place 1 gram in 100 c.c. of sterile distilled water and boil with a condenser for 2 hours; or boil in an Arnold sterilizer for the same time, making up for any loss in volume by adding distilled water.

5. Add 2 grams of sodium chloride and 10 c.c. of 5% phenol or tricresol. Place in a tightly stoppered bottle in a refrigerator to ripen for a week before titrating.

In case one so desires smaller quantities can readily be made up in similar proportions.

The Price Method for Preparing Gonococcus Antigen.—Gonococci, grown on hydrocele agar (pH 7.5) in a triangular Roux bottle at 37.5° C. for 24-48 hours, are washed off into a cylinder with 100 c.c. of "physiological" saline (0.9%); this usually yields a suspension of about 180 millions of organisms per c.c. One c.c. of N/1 NaOH is added, and the cylinder placed in the 37.5° C. bath for 2 hours, after which most of the organisms will be found to be in solution. The fluid is then

filtered through sterile lint to remove pieces of medium and other, gross insoluble matter. To the clear filtrate 1.5 c.c. of N/1 HCl is now added, and the cylinder returned to the bath. After 10 to 20 minutes, white flocculi appear, and these are centrifuged out of solution (3,000 r.p.m.) and then suspended in 4 c.c. of sterile saline. N/10 NaOH is added to the suspension. drop by drop, until a pH of 7.5 to phenol red is reached. By vigorous shaking. the suspension appears to go into solution. No further addition of alkali affects the solubility of the precipitate, but if the alkaline reaction is carried above pH 9.5 its antigenic properties become definitely weaker; 1 c.c. of 1% formalin is added and the "solution" is then filtered through sterile lint. This colloidal solution constitutes the concentrated "antigen" and for use in the test proper it is diluted according to its titer. The antigen obtained in the manner described is not a true solution, as by centrifugalization the colloidal suspension of protein can be thrown down as a deposit. It is advisable to prepare the antigen in bulk, using up to 10 Roux bottles; a sample of antigen is prepared separately from each bottle according to the above technic. Each sample having been formalized, they are mixed and the mixture is filtered through sterile lint. The resulting filtrate is transferred into sterile ampules or test tubes (5 c.c. capacity) which are hermetically sealed and then heated for 2 hours at 56° C. Before use the titer of the antigen of a sample ampule is ascertained, and once this titer is known no subsequent titration is necessary. It is important that the test tube or ampule containing the concentrated antigen should be well shaken before measuring out the quantity (indicated by its titer) required for dilution. The antigen can be kept on ice or at room temperature, and, provided no contamination has occurred, it will keep its properties indefinitely.

Salmonella Pullorum (Bacillary White Diarrhea of Chickens).—METHOD OF BUSHNELL AND HUDSON.—Cultivate *Salmonella pullorum* on agar. Wash off with saline solution. Shake vigorously for several minutes and filter through glass wool. Centrifuge for 45 to 60 minutes at high speed. Discard the supernatant fluid. To each c.c. of sediment add 10 c.c. of ether. Mix well for 4 hours. Discard the ether. Add fresh ether and extract for 2 hours. Discard ether and dry residue in incubator. Suspend the residue in sufficient saline solution to give a turbidity equal to tube 3 of the McFarland nephelometer.

Brucella Abortus (Bovine Infectious Abortion).—METHOD OF BOERNER AND STUBBS.—Cultivate several strains on liver infusion or glycerin agar for 4 to 7 days. Wash off with small amounts of sterile distilled water. Heat at 100° C. for 3 hours and place in refrigerator for 10 days with occasional shakings. Then shake well and centrifuge at low speed for a short time to throw down a greater portion of the bacteria. The supernatant fluid, which should be still quite turbid, is pipeted off; add phenol to 0.5%. Keep in refrigerator.

Titration of Bacterial Antigens.—The technic is exactly the same as described for titration of antigen for the syphilis reaction except that the dilutions are slightly different.

1. The following table gives directions for preparing the dilutions and it makes no difference whether antigen is added to saline or saline to antigen:

2.0 c.c. antigen (full strength) + 2.0 c.c. saline solution = 1 : 2
1.0 c.c. antigen (full strength) + 2.0 c.c. saline solution = 1 : 3
1.0 c.c. antigen (1 : 2)   + 1.0 c.c. saline solution = 1 : 4
1.0 c.c. antigen (1 : 3)   + 1.0 c.c. saline solution = 1 : 6
1.0 c.c. antigen (1 : 4)   + 1.0 c.c. saline solution = 1 : 8
0.5 c.c. antigen (1 : 2)   + 2.0 c.c. saline solution = 1 : 10
1.0 c.c. antigen (1 : 6)   + 1.0 c.c. saline solution = 1 : 12
1.0 c.c. antigen (1 : 8)   + 1.0 c.c. saline solution = 1 : 16
1.0 c.c. antigen (1 : 10)   + 1.0 c.c. saline solution = 1 : 20
0.2 c.c. antigen (1 : 4)   + 1.8 c.c. saline solution = 1 : 40
0.2 c.c. antigen (1 : 6)   + 1.8 c.c. saline solution = 1 : 60
0.2 c.c. antigen (1 : 8)   + 1.8 c.c. saline solution = 1 : 80
0.2 c.c. antigen (1 : 10)   + 1.8 c.c. saline solution = 1 : 100
0.2 c.c. antigen (1 : 12)   + 1.8 c.c. saline solution = 1 : 120
1.0 c.c. antigen (1 : 80)   + 1.0 c.c. saline solution = 1 : 160
1.0 c.c. antigen (1 : 100)   + 1.0 c.c. saline solution = 1 : 200
0.5 c.c. antigen (1 : 100)   + 1.0 c.c. saline solution = 1 : 300
0.5 c.c. antigen (1 : 200)   + 1.0 c.c. saline solution = 1 : 400

2. The following table shows the method of titrating for *hemolytic activity*, but since bacterial antigens prepared by the methods given above are rarely hemolytic, this titration may be safely omitted:

Tube	Antigen, 0.5 c.c.	Heated Human Serum * c c (1 /10)	Saline Solution, c.c.	6° to 8° C. for 15 to 18 hours, followed by 30 minutes in water bath	Corpuscles, c.c. (2 per cent)	Water bath for one hour	Example of Readings
1	Full strength	0.5	1.5		0.5		Slight hemolysis†
2	1:2	0.5	1.5		0.5		No hemolysis
3	1:3	0.5	1.5		0.5		No hemolysis
4	1:4	0.5	1.5		0.5		No hemolysis
5	1:6	0.5	1.5		0.5		No hemolysis
6	1:8	0.5	1.5		0.5		No hemolysis

* May be omitted, in which case 2 c c saline are added to each tube instead of 1 5 c c.
† Tubes containing large amounts of antigen should be centrifuged to determine any hemolysis

The *hemolytic unit* is the smallest amount of antigen just producing some hemolysis; in the above table this is 0.5 c.c. of full strength.

3. The first table given on page 641 shows the method of titrating for *anti-complementary activity* and is the most important of all; it must be conducted at frequent intervals with most bacterial antigens.

The *anticomplementary unit* is the smallest amount of antigen giving slight inhibition of hemolysis; in the above table this is 0.5 c.c. of 1:6 dilution.

4. The second table given on page 641 shows the method of titrating for *antigenic activity*, which is not essential but always advisable if a suitable positive serum is available.

The *antigenic unit* is the smallest amount of antigen giving + + + + or complete inhibition of hemolysin. In the above table this is 0.5 c.c. of 1:60 dilution.

ANTICOMPLEMENTARY TITRATION

Tube	Antigen, 0 5 c c	Healed Human Serum,* c c (1·10)	Complement, c c (2 full units)	Refrigerator at 6° to 8° C. for 15 to 18 hours, followed by water bath for 30 minutes; or water bath only for 2 hours	Hemolysin, c c. (2 units)	Corpuscles, c c (2 per cent)	Water bath for one hour	Example of Readings
1	Full strength	0.5	1.0		0.5	0.5		No hemolysis
2	1:2	0.5	1.0		0.5	0.5		No hemolysis
3	1:3	0.5	1.0		0.5	0.5		Slight hemolysis
4	1:4	0.5	1.0		0.5	0.5		Slight hemolysis
5	1:6	0.5	1.0		0.5	0.5		Marked hemolysis†
6	1:8	0.5	1.0		0.5	0.5		Complete hemolysis
7	1:10	0.5	1.0		0.5	0.5		Complete hemolysis
8	1:12	0.5	1.0		0.5	0.5		Complete hemolysis
9	1:16	0.5	1.0		0.5	0.5		Complete hemolysis
10	1:20	0.5	1.0		0.5	0.5		Complete hemolysis
11	None; 0.5 c.c. saline solution	0.5	1.0		0.5	0.5		Complete hemolysis‡
12	None; 2.5 c.c. saline solution	None	None		None	0.5		No hemolysis§

* May be omitted and 0 5 c c of saline added instead
† Anticomplementary unit being smallest amount of antigen giving slight inhibition of hemolysis
‡ Hemolytic system and serum control
§ Corpuscle control

ANTIGENIC TITRATION

Tube	Antigen, 0 5 c c.	Healed Positive Serum * c c (1·10)	Complement, c c (2 full units)	Refrigerator at 6° to 8° C. for 15 to 18 hours, followed by water bath for 30 minutes; or water bath only for 2 hours	Hemolysin, c c (2 units)	Corpuscles, c.c (2 per cent)	Water bath for one hour	Examples of Readings
1	1:10	0.5	1.0		0.5	0.5		++++
2	1:20	0.5	1.0		0.5	0 5		++++
3	1:40	0.5	1.0		0.5	0.5		++++
4	1:60	0.5	1.0		0.5	0.5		++++ (†)
5	1:80	0.5	1.0		0.5	0.5		+++
6	1:100	0.5	1.0		0.5	0.5		+++
7	1:120	0.5	1.0		0.5	0.5		++
8	1:200	0.5	1.0		0.5	0.5		+
9	1:300	0.5	1.0		0.5	0.5		−
10	1:400	0.5	1.0		0.5	0.5		−
11	0.5 c.c. Saline solution 1.0 c.c.	0.5	1.0		0.5	0.5		−‡
12	Saline solution	None	1.0		0.5	0.5		−§

* 0 5 c c of 1:10 dilution human positive serum heated at 55° C for 15 to 20 minutes, same if horse or guinea-pig immune sera are used, cattle sera 58° to 60° C for 30 minutes; mule sera 62° C for 30 minutes   But 0 5 c c. of 1·50 if a rabbit immune serum (he ted at 60° to 62° C for 30 minutes) is being used in the above titration.
† The antigenic unit being the smallest amount of antigen giving a ++++ reaction.
‡ Serum control giving complete hemolysis
§ Hemolytic system control giving complete hemolysis

The Dose of Antigen to Employ.—*A dose of bacterial antigen equivalent to one-third of the anticomplementary units is used.* For example, if the anticomplementary unit of an antigen happens to be 0.5 c.c. of 1 : 6 dilution, the dose may be 0.5 c.c. of 1 : 18, and the amount would probably carry 2 to 10 antigenic units as determined by the antigenic titration.

The antigenic titration is always advisable if a suitable positive serum is available, but if not, the anticomplementary titration alone may be made and the antigen used in an *arbitrary dose* equivalent to one-third of the anticomplementary unit.

Bacterial antigens keep very well in the refrigerator; it generally suffices to *titrate but once a month*, although one may titrate for anticomplementary activity each time tests are conducted by using the 2-hour water bath incubation.

Quantitative and Qualitative Complement-Fixation Tests.—The technic of these is exactly as described for the syphilis tests except that the primary incubation may be either (*a*) 15 to 18 hours in the refrigerator at 6° to 8° C. followed by ½ hour in a water bath at 38° C. or (*b*) 2 hours in a water bath. The latter is particularly recommended for the tuberculosis complement-fixation test. In the *quantitative test* serum is used in amounts of 0.2, 0.1, 0.05, 0.025 and 0.005 c.c. with 0.2 c.c. in the control; in the *qualitative* test the serum is used in amounts of 0.2 and 0.1 c.c. with 0.2 c.c. in the control.

It is advisable to include positive and negative controls, especially the former. The negative controls should be of human sera in the tuberculosis, gonococcus, typhoid and such tests; the positive controls should be of human sera when available but otherwise immune sera of the lower animals may be used.

The readings should be made 10 minutes after complete hemolysis of the antigen control in the case of those sera showing complete hemolysis of the serum controls; these give the most sensitive readings. Otherwise the readings should be made immediately after the secondary incubation of one hour, providing the antigen, serum, hemolytic system and negative serum controls show complete hemolysis.

In conducting the tuberculosis complement-fixation test, the Wassermann test should be always conducted at the same time because syphilis antibody may give a positive reaction with tuberculosis antigen in the absence of tuberculosis. When the Wassermann reaction is strongly positive, the tuberculosis test is also quite apt to yield a positive reaction and should be reported upon with great caution. This is not true, however, in the case of the gonococcus, typhoid and other bacterial complement-fixation tests.

KOLMER COMPLEMENT-FIXATION TESTS FOR TRYPANOSOMIASIS AND ECHINOCOCCUS DISEASE

Methods for preparing the various antigens are given below. The methods for titrating the hemolytic, anticomplementary and antigenic activities are exactly as given for the titration of bacterial antigens, the hemolytic system and general technic being exactly as described for syphilis.

Each antigen is employed in a dose equivalent to one-third of its anticomplementary unit since the large amounts yield the most sensitive reactions, prozone reactions being quite uncommon. Therefore the anticomplementary unit of each antigen must be known on the basis of preliminary titrations.

In conducting the main tests, either the quantitative or qualitative technic may be employed with a primary incubation of 15 to 18 hours in a refrigerator at 6° to 8° C. followed by ½ hour in a water bath at 38° C., or the water bath only may be employed for 2 hours; the former has yielded the more sensitive and satisfactory results.

In conducting the echinococcus and other tests with human sera, it is advisable

(indeed necessary) to conduct a Wassermann test at the same time because all of these antigens are capable of yielding cross-complement-fixation reactions with sera containing large amounts of syphilis antibody. Whenever a serum gives a positive Wassermann reaction, the results of positive echinococcus or other reactions should be interpreted with due care.

When rabbit immune sera are used for positive controls, the dose should not be more than 0.5 c.c. of 1:50 dilution (0.01 c.c. serum) in order to avoid the nonspecific reactions which are sometimes yielded by normal rabbit serum with these antigens.

Antigens of Trypanosomes.—As a general rule these are prepared of *Trypanosoma equiperdum* for complement-fixation tests with the sera of horses for dourine.

White rats are inoculated and as soon as the tail blood shows the presence of a heavy infection, antigens are prepared by securing the organisms from the blood by the method of Reynolds and Schoening as follows: "Blood of infected rats is collected in a 1% sodium citrate solution in physiological salt solution in order to prevent coagulation. When all the blood has been collected, the solution is filtered through cheesecloth to remove clots, fibrin, etc., poured into tubes, and centrifugalized for about 20 minutes at 2100 revolutions per minute. This precipitates all the corpuscles and most of the trypanosomes, leaving an upper stratum of blood serum and citrate solution containing some of the organisms. This fluid is drawn off and again centrifugalized in order to recover any of the protozoa which may be present. To the other tubes containing the mass of corpuscles intermixed with and superimposed by trypanosomes is added sufficient distilled water to produce complete hemolysis of the rat erythrocytes, a matter of about 20 minutes, which procedure is facilitated by agitation of the mixture in a flask. This also is centrifugalized but in this instance for about ½ hour, upon the completion of which there is found at the bottom of the tubes a mass of trypanosomes with an admixture of stroma of the hemolyzed red cells, which latter, in quantity, has been found to be negligible. After discarding the supernatant fluid (hemoglobin-stained water) physiological salt solution is added and the material vigorously shaken until the mass of trypanosomes is disintegrated and evenly distributed throughout the solution. Centrifuging is again resorted to with similar results, the washed mass of trypanosomes being packed at the bottom of the tubes. The salt solution is poured off and an amount of preserving fluid (physiological salt solution and glycerin) equal to about twice the amount of trypanosomes added; the mixture is then agitated until a uniform suspension is acquired, when it is stored at a low temperature until used."

Echinococcus Antigen.—The fluid from echinococcus cysts has been usually employed preserved with 0.5% phenol in a refrigerator. It would appear, however, that the scolices contain most of the antigenic principles and a better antigen may be prepared by grinding up the moist scolices with fine sand in a mortar and adding 9 volumes of the clear cyst fluid or saline solution to give an approximate 10% extract of the scolices. Phenol or tricresol should be added to 0.25% and the

mixture extracted in an incubator at 37° C. for 4 days, filtered, and stored in a refrigerator.

Fairley recommends an alcoholic extract prepared by grinding the scolices with fine sand, adding 9 volumes of absolute ethyl alcohol to give a 10% extract, and placing the mixture in an incubator for 2 days when it is filtered and stored for use.

KOLMER COMPLEMENT-FIXATION TESTS WITH RABBIT, DOG AND MULE SERA

The sera of some of the lower animals, notably of the rabbit, dog, and mule, sometimes yield nonspecific complement-fixation and precipitation reactions with the various antigens employed in the syphilis reactions. With various bacterial antigens the degree of fixation is even greater. *Therefore, in conducting complement-fixation reactions with the sera of these animals, the technic must be modified to avoid the possibility of these nonspecific reactions and yet sufficiently sensitive for the detection of specific antibody.* These ends are met by heating the sera at 62° C. instead of at 55° to 56° C. (first method) and by using more complement (first method) or smaller doses of serum (second method).

First Method.—*The technic is exactly the same as described for the testing of human sera except:*

1. The natural antisheep hemolysins are not removed from the sera.

2. The sera are heated in a water bath at 62° C. for 30 minutes.

3. The doses in *syphilis* tests are 0.1, 0.05, 0.025, 0.0125, 0.006 and 0.1 c.c. (control) in the quantitative test. But in *bacterial* complement-fixation tests smaller amounts of serum should be used as:

$$0.025 \text{ c.c. } (0.5 \text{ c.c. of } 1:20)$$
$$0.012 \text{ c.c. } (0.5 \text{ c.c. of } 1:40)$$
$$0.006 \text{ c.c. } (0.5 \text{ c.c. of } 1:80)$$
$$0.003 \text{ c.c. } (0.5 \text{ c.c. of } 1:160), \text{ etc.}$$
$$0.025 \text{ c.c. } (0.5 \text{ c.c. of } 1:20) \text{ control}$$

The first two doses of either series are used in the qualitative test, i.e., 0.1, 0.05 and 0.1 c.c. (control) with cholesterolized and lecithinized alcoholic extract of beef heart antigen in syphilis tests, or 0.025, 0.012 and 0.025 c.c. (control) in bacterial complement-fixation tests employing the antigen in a dose equivalent to *one-fourth* of its anticomplementary unit.

4. *Four units* of complement are used instead of 2½ and so diluted that this dose is contained in 1 c.c. Example:

$$\text{Unit} = 0.3 \text{ c.c. of } 1:30$$
$$\text{Four units} = 1.2 \text{ c.c. of } 1:30$$

To calculate the dilution to use so that 1 c.c. contains the dose, divide 30 by the dose:

$$\frac{30}{1.2} = 25 \text{ or dilution } 1:25 \text{ in dose of } 1 \text{ c.c.}$$

Second Method.—_The technic is exactly the same as described for testing human sera_ except:

1. The natural antisheep hemolysins are not removed from the sera.
2. The sera are heated in a water bath at 62° C. for 30 minutes.
3. The doses of serum in quantitative *syphilis* tests with the usual dose of antigen are:

> 0.025 c.c. (0.5 c.c. of 1 : 20)
> 0.0125 c.c. (0.5 c.c. of 1 : 40)
> 0.006 c.c. (0.5 c.c. of 1 : 80)
> 0.003 c.c. (0.5 c.c. of 1 : 160)
> 0.0015 c.c. (0.5 c.c. of 1 : 320)
> 0.025 c.c. (0.5 c.c. of 1 : 20) control

The doses in quantitative *bacterial* tests with one-fourth of the anticomplementary unit of antigen are:

> 0.0125 c.c. (0.5 c.c. of 1 : 40)
> 0.006 c.c. (0.5 c.c. of 1 : 80)
> 0.003 c.c. (0.5 c.c. of 1 : 160)
> 0.0015 c.c. (0.5 c.c. of 1 : 320)
> 0.0008 c.c. (0.5 c.c. of 1 : 640)
> 0.0125 c.c. (0.5 c.c. of 1 : 40) control

In qualitative tests the first 2 doses of either series are employed along with the larger amount in the third tube or serum control.

Heating sera at 62° C. for 30 minutes does not destroy enough antibody in syphilitic rabbits or in rabbits and dogs immunized to various antigens to interfere with the sensitiveness of the reactions; nor does this degree of heating of mule sera interfere with the sensitiveness of the glanders complement-fixation test for which they are usually submitted.

KOLMER COMPLEMENT-FIXATION TESTS WITH URINE, MILK, TRANSUDATES AND EXUDATES

Transudates like pleural, pericardial, peritoneal and joint fluids are usually free of anticomplementary activity and may be tested in the same manner as serum. They should be heated at 55° C. for 15 minutes. As a general rule, however, their antibody content is less than in serum and larger doses are sometimes required similar to those employed in testing spinal fluid (0.5, 0.25, 0.125 c.c., etc.).

Exudates like blister fluids and tuberculous pleural exudates are much more likely to be anticomplementary; likewise urine and milk.

Urine, milk and *exudates* (like pleural exudates for tuberculosis) should be freshly collected and kept at a low temperature until examined. Each should be heated at 55° C. for 15 minutes and first tested for anticomplementary activity as follows:

1. Titrate hemolysin.

2. In a series of eight test tubes place 0.5 c.c. undiluted, 1:2, 1:3, 1:4, 1:6, 1:8, 1:12 and 1:16 dilutions of the fluid to be tested. Titrate the complement and add 2 full units (1 c.c.); also 0.5 c.c. of saline solution.

3. Water bath at 37° C. for 1 hour.

4. Add 2 units of hemolysin and 0.5 c.c. of 2% corpuscles.

5. Water bath 1 hour and read.

6. Include a hemolytic system and corpuscle control.

The smallest amount giving even slight interference of hemolysis is the anti-complementary unit and varies greatly with different specimens.

7. In setting up the complement-fixation tests, place one-fourth of the anti-complementary unit in a front and rear tube; for example, if this happens to be 0.5 c.c. of 1:4 dilution, use 0.5 c.c. of 1:16.

8. To the front tube add the usual dose of antigen and to the rear tube 0.5 c.c. of saline (control).

9. The complement is titrated in the presence of the antigen in the usual manner and 2 full units added to both tubes.

10. The primary incubation is the usual 15 to 18 hours at 6° to 8° followed by 10 to 15 minutes in a water bath, the test being finished in the usual way.

CHAPTER XXX

METHODS FOR CONDUCTING PRECIPITATION TESTS

Numerous precipitation or flocculation tests have been described for the serum diagnosis of syphilis by Michaelis, Sachs-Georgi, Vernes, Meinicke, Dreyer and Ward, Kahn, Kline, Eagle, Hinton, Rosenthal and others. Their value in comparison to the Wassermann test depends not so much on their infallibility as on the fact that each technic affords a different approach to the serum diagnosis of syphilis and to the serological guidance of treatment.

KAHN TEST FOR SYPHILIS [1]

Standard Apparatus.—1. Antigen suspension vials: 5.5 centimeters in length and 1.5 centimeters in inside diameter (1.7 centimeters outside diameter).

2. Test tubes: 7.5 centimeters in length and 1 centimeter in inside diameter (1.2 centimeters outside diameter).

3. Pipets for measuring antigen suspension: (*a*) 1.5 c.c., 1 c.c. or 0.5 c.c.
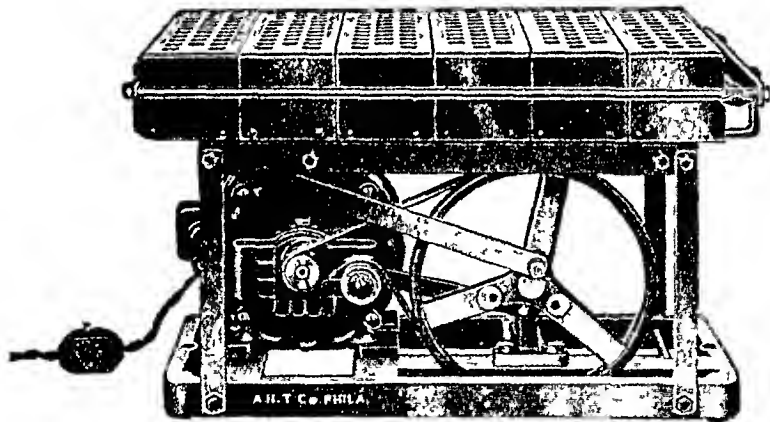
Fig. 340.—KAHN SHAKING APPARATUS

graduated to 0.05 c.c., for measuring the 0.05 c.c. quantities of suspension; (*b*) 0.25 c.c. graduated to 0.0125 c.c. or 0.2 c.c. graduated to 0.001 c.c., for measuring the 0.025 and 0.0125 c.c. amounts of antigen suspension.

4. Pipets for measuring serum: 1 c.c. graduated to 0.01 c.c. or 0.45 c.c. (or 0.6 c.c.) graduated to 0.15 c.c.

5. Pipets for measuring saline: (*a*) 10 c.c. graduated to 0.1 c.c.; (*b*) 1 c.c. or 2 c.c. graduated to 0.01 c.c.

[1] For a more detailed description of the test consult *The Kahn Test—A Practical Guide*, Williams and Wilkens Company, Baltimore, 1928.

6. Test tube racks: These racks are made of sheet copper and are 3 inches wide, 11½ inch long and 2¾ inches high. They are constructed of 3 shelves, upper and middle ones containing 3 rows of 10 holes, each of approximately ½ inch in diameter. The holes of the center row are offset ½ inch. The bottom shelf serves as a support.

7. Shaking apparatus: There are several types of shaking apparatus available on the market. Most shakers are constructed to hold a maximum number of 6 racks (Fig. 340) and are adjustable to hold less than this number if it is desired. Other shakers are constructed with a capacity for holding less than 6 racks, for use in laboratories where small numbers of tests are performed. The standard speed ranges from 275 to 285 oscillations per minute, with a stroke of 1½ inches. The speed should be checked from time to time to assure conformity to this requirement. It may be desirable to enclose the 6-rack shaker in a wooden box to reduce the noise during the shaking period. Such a box should be lined with felt or some similar material which will absorb sound.

*Preparation of Reagents.—Serum.—*Collect blood as for the complement-fixation test; separate the sera and heat at 56° C. for 30 minutes.

*Saline Solution.—*Dissolve 9 grams of chemically pure sodium chloride in 1000 c.c. of distilled water and filter.

*Antigen.—*The antigen for the Kahn test is a specially prepared alcoholic extract (cholesterolized) of powdered beef heart from which the ether-soluble elements have been partially removed.

Powdered beef heart for preparing antigen is now obtainable on the market in the form of Bacto-Beef Heart, which gives highly uniform results, due undoubtedly to the fact that a large number of hearts are used in making a given lot. Because of this uniformity, and also because of the labor- and time-saving factors, it is advantageous to employ this product in the preparation of antigen.

1. Fifty grams[2] of powdered beef heart are placed in a 500 c.c. Erlenmeyer flask. Two hundred c.c. of ether (anesthesia) are added and the flask is shaken at frequent intervals for 10 minutes. At the end of this period the ether is filtered off. Gentle pressure is applied to the beef heart in the funnel by means of a spatula, to assure as complete removal of the ether as possible. The filtration is completed when practically no drops of ether pass through the funnel as a result of pressure with the spatula.

2. The moist beef heart is transferred to the original flask. This may be done by first transferring the beef heart from the funnel to a sheet of white paper and breaking the material with a spatula into pieces small enough for the mouth of the flask. One hundred and fifty c.c. of ether are added to the flask, which is again shaken at frequent intervals during a 10-minute period. The ether is then filtered off as in the previous case.

3. The heart muscle is returned to the flask a third time and again covered with

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[2] When employing 100 gms. of beef heart, use 1000 c.c. Erlenmeyer flask and double the amounts of ether as compared with the amounts used for 50 gms.

150 c.c. of ether. The flask is shaken from time to time during a 10-minute period and filtration carried out as previously.

4. The moist powder is then transferred to the Erlenmeyer flask for the fourth and last ether extraction. One hundred and fifty c.c. of ether are added to the flask and after a 10-minute extraction period with frequent shaking, the final filtration of the ether is carried out. It is well to employ fresh filter paper for each filtration, but care should be taken to minimize the loss of powdered muscle by scraping as much of the residue as possible from the paper into the extraction flask. When the moist heart muscle has been freed from ether as completely as in the earlier filtrations, it is spread upon a sheet of white paper or a clean glass plate and dried for about 30 minutes, at room temperature. When the material is dry and free from ether odor, it is ready for extraction with alcohol. The same Erlenmeyer flask in which the ether extraction was carried out may be employed for the alcohol extraction, provided the ether has been completely removed before the adding of the powdered muscle and alcohol.

5. After completing the ether extractions, the dried powder is weighed and transferred to a 500 c.c. Erlenmeyer flask. Five c.c. of 95% alcohol are added per gram of powder, the flask is shaken for 10 minutes, and extraction continued for 3 days at room temperature about 21° C. The flask is not shaken again during this extraction period except for a 5-minute period just before filtration. The alcoholic extract after filtration is kept at room temperature in the dark as stock solution. All corks employed in connection with the preparation and storing of antigen are covered with high-grade thin tin foil.

6. A given amount of the alcoholic extract is measured into an Erlenmeyer flask and 6 milligrams of cholesterol are added per c.c. of extract. The flask is placed in warm water bath and rotated to hasten solution of the cholesterol. When the latter is entirely dissolved, the antigen is filtered and is ready for standardization.

Titration of Antigen.—1. Measure 0.8, 1.0, 1.1, 1.2, 1.3 c.c. respectively of physiological salt solution into five standard antigen suspension vials (5.5 centimeters length, 1.5 centimeters diameter).

2. Measure into each of 5 similar vials 1 c.c. of cholesterolized antigen.

3. Prepare 5 antigen suspensions by mixing the 1 c.c. quantities of antigen with the varying amounts of salt solution, in series. Empty the salt solution into the antigen and as rapidly as possible (without waiting to drain the tube) pour the mixture back and forth 6 times. Permit the mixture to stand for ten and not over thirty minutes.

4. Test for the dispersability in salt solution of the lipid aggregates present in the antigen-salt solution suspensions after thoroughly mixing as follows:

(a) Set up 5 series of 3 standard tubes (employed in performing the regular Kahn test with serum, 7.5 centimeters length, 1 centimeter diameter).

(b) Pipet 0.05, 0.025, and 0.0125 c.c. quantities of each of the 5 antigen suspensions, in series, to the bottom of the tubes, using a 0.2 or 0.25 c.c. pipet marked in 0.001 or 0.0125 c.c. amounts.

When measuring the antigen suspensions in series, it is advisable to begin with the suspension containing the largest amount of salt solution, and end with the one containing the least amount of salt solution. This will avoid carrying non-dispersable lipid aggregates from one suspension to the other.

(c) Add 0.15 c.c. salt solution to each of the 15 tubes.

(d) Shake the rack of tubes for 3 minutes in a shaking apparatus at a speed of 275 to 285 oscillations per minute. If no such apparatus is available, rapid shaking by hand will approximate this speed.

(e) Add 1 c.c. salt solution to the tubes containing the 0.05 c.c. amounts of antigen suspension, and 0.5 c.c. to the remaining tubes. Observe whether fluids are opalescent or contain aggregates.

Interpretation of Results.—When each of the 5 antigen suspensions are thus tested for the dispersability of aggregates, it may be found that the antigen suspensions prepared by mixing antigen with the smallest amounts of salt solution contain aggregates which are not completely dispersed in additional salt solution. The titer of the antigen is the smallest amount of salt solution which, when added to 1 c.c. antigen, produces aggregates capable of complete dispersion upon the addition of further salt solution and giving an opalescent medium which is free from cholesterol crystals. If 1.3 c.c. salt solution added to 1 c.c. antigen results in a suspension containing particles that are not dispersed in additional salt solution, the titration is continued with volumes greater than 1.3 c.c. A titer higher than $1 + 1.5$ can usually be avoided by a modification of the antigen itself.

Determination of Sensitiveness of Antigen.—The sensitiveness of a new antigen is determined by comparing it with "standard Kahn antigen." The degree of sensitiveness of "standard Kahn antigen" was established to give as high a percentage of specific positive reactions as was possible without giving non-specific reactions. Each new antigen that is brought to that established degree of sensitiveness becomes a "standard Kahn antigen."

1. Preparation of Syphilitic Sera for Comparative Tests.—Ten sera are obtained, 8 from syphilitic patients and 2 from nonsyphilitic individuals. Of the 8 sera, at least 6 should give weakly positive reactions, and the remaining, strongly positive reactions. Pooled sera may be employed. All sera are heated for 30 minutes at 56° C. before being tested. If the sera employed have been previously heated for 30 minutes, they should be reheated for 10 minutes at the same temperature before use.

2. Testing Newly Prepared and Standard Antigen with Sera.—Antigen suspensions are prepared with both antigens in accordance with their respective titers. Both suspensions are permitted to stand for 10 minutes and each is pipeted in 0.05, 0.025, and 0.0125 c.c. amounts for a series of 10 Kahn tests. The sera are then added in 0.15 c.c. amounts. All the tests are shaken for 3 minutes at 275 oscillations per minute and after adding the proper amounts of salt solution to each tube, the results with the two antigens are compared.

3. Interpretation of Results.—If the results of the comparative tests with the 2 antigens are closely comparable, the new antigen probably possesses standard sensitiveness. To eliminate the possibility of error, at least 2 additional series of

comparative tests are carried out, and if the results are again comparable the newly prepared antigen may be considered as standard, although it is desirable to make as many comparative tests as possible with nonpooled sera before declaring an antigen standard.

Correction of Antigen.—The sensitiveness of a newly prepared antigen may be greater or less than that of standard antigen. In either case it can readily be corrected to standard requirements. Two reagents are necessary for antigen correction: cholesterolized alcohol and sensitizing reagent.

Preparation of Cholesterolized Alcohol.—Cholesterolized alcohol is prepared similarly to cholesterolized antigen. Thus, for cholesterolizing 100 c.c. of 95% alcohol the alcohol is added to 600 milligrams of cholesterol in a 250 c.c. Erlenmeyer flask or similar container. The cork to be employed should be covered with thin, high-grade tin foil. Rotate flask in a warm water bath until all cholesterol is dissolved. Filter to remove traces of foreign material. The solution is then ready for use.

Preparation of Sensitizing Reagent.—1. The ether filtrate obtained in the preparation of antigen from 50 grams of heart muscle is refiltered to remove traces of powdered muscle, and is then evaporated with the aid of an electric fan.

2. When the volume has been reduced to about 10 c.c. or less, the concentrated ether extract is transferred to a small, weighed evaporating dish (capacity about 25 c.c.), the transfer being made complete by washing out the residue into the small dish with a little ether.

3. Evaporation is continued with the aid of the fan until the ether odor is no longer detectable.

4. At this stage there may separate from the dark brown lipid mass, a few c.c. of water. This water, which will be at the bottom of the evaporating dish, is removed by means of a capillary pipet. The lipid residue is brownish, semi-transparent and viscous.

5. The evaporating dish is now reweighed, and the weight of the residue determined.

6. The residue is transferred to an Erlenmeyer flask (about 100 c.c. capacity). This is best accomplished with the aid of a small spatula.

7. A volume of absolute alcohol equivalent to 10 c.c. per gram of residue is added to the flask. A small amount of this alcohol is employed for rinsing the evaporating dish.

8. Extraction is allowed to take place for 30 minutes at room temperature with frequent shaking of the flask.

9. The mixture is filtered, and the filtrate is allowed to stand at room temperature for three days. If a precipitate forms during this period, the solution is refiltered.

10. The filtrate is cholesterolized with 6 mgm. cholesterol per cubic centimeter, according to the usual technic.

11. The cholesterolized extract known as "sensitizing reagent" is filtered and is ready for use.

Methods of Correction.—In order to understand the methods of correcting antigen to "standard" sensitiveness, it is necessary to recall that antigen sensitiveness (according to Kahn) is directly related to the concentration of lipids in the antigen. Only at a certain lipid concentration does an antigen give maximum sensitiveness, while excessive or deficient concentration reduces antigen sensitiveness. Furthermore, the degree of sensitiveness of standard Kahn antigen does not represent the maximum sensitiveness of which an antigen is capable, but instead represents a definitely chosen conservative degree of sensitiveness in conformity with specificity. Some newly prepared antigens will thus be more and some less sensitive than standard antigen, depending on their concentration of antigenic lipids.

ANTIGENS MORE SENSITIVE THAN STANDARD ANTIGEN.—When an antigen is more sensitive than standard antigen, it could be corrected either by concentrating the lipids of the antigen or by diluting the antigen with cholesterolized alcohol (since excessive concentration or dilution reduces antigen sensitiveness).

For simplicity, the method of choice is that of dilution. Technic: To a small amount of the oversensitive antigen, such as 10 c.c., is added 1.5 c.c. cholesterolized alcohol (15% dilution). The diluted antigen is now tested against the standard antigen, using weakly positive sera. If comparable, the entire lot of new antigen is diluted with 15% of cholesterolized alcohol. If not comparable, then if the antigen after 15% dilution is still more sensitive than standard, a higher dilution, such as 25%, is tried; if 15% dilution reduced the sensitiveness below that of standard, a lesser dilution, such as 10%, is tried.

The method of lipid concentration for reducing antigen sensitiveness is resorted to only when it is found that an antigen requires an excessive dilution (beyond 25%) of cholesterolized alcohol to bring the sensitiveness to standard requirements. Technic: 1.5 c.c. of noncholesterolized antigen is placed in a small evaporating dish and evaporated to dryness by means of an electric fan. The lipid residue is taken up in 10 c.c. of the oversensitive antigen (15% concentration). The concentrated antigen is now tested against the standard antigen as above. If the new antigen is still more sensitive than standard, a higher concentration, such as 25%, is tried; if less sensitive than standard, the concentration is reduced to perhaps 10%.

ANTIGEN LESS SENSITIVE THAN STANDARD ANTIGEN.—An antigen is less sensitive than standard when it is either too rich or too poor in lipids. An antigen which is less sensitive than standard because of excessive lipid concentration is corrected by dilution with cholesterolized alcohol, employing the identical technic described above for correcting oversensitive antigens. An antigen less sensitive than standard due to insufficient lipid concentration is corrected by adding a small amount of sensitizing reagent, such as 0.5%. Technic: To 10 c.c. of the less sensitive antigen is added 0.05 c.c. of sensitizing reagent. The modified antigen is now compared with standard antigen in the usual way. If still less sensitive than standard, the amount of sensitizing reagent is increased to 1 or more per cent; if more sensitive, the amount of reagent is reduced below 0.5%.

Varying the Amount of Salt Solution in the Titer.—An antigen is most sensitive when mixed with salt solution according to its titer. If the titer of an antigen. let us say, is $1+1.1$, then antigen sensitiveness is gradually reduced by employing antigen suspensions of $1+1.2$, $1+1.3$, etc. Therefore, an antigen having a titer of $1+1.1$ and more sensitive than standard antigen could obviously be brought down to standard requirements by employing titers of $1+1.3$, $1+1.5$ or even greater amounts of salt solution. In doing this, however, care must be taken not to reduce the opalescence of the completed reactions to the point where difficulty is encountered in reading results. Since most studies on the Kahn reaction have been carried out with antigen titers ranging from $1+1$ to $1+1.3$, Kahn does not recommend the use of larger amounts of salt solution in the titer, and prefers reducing the sensitiveness of antigens to standard requirements by modifying the lipoidal content of the antigen.

The information gained from the standardization of the small sample of antigen may be utilized in the standardization of the entire amount of it, after which comparative tests with at least 40 sera should be made. If the comparison is favorable, the new antigen may be considered as of standard sensitiveness.

Performance of the Standard Test.—It is well to have the necessary equipment for the test ready before preparing the antigen suspension. Have racks set up, tubes numbered, sera heated and pipets ready for measuring the antigen suspension and serum. For measuring the 0.05 c.c. quantities of antigen suspension, a 0.5, 1.0 or 1.5 c.c. pipet may be employed, graduated in 0.05 c.c. amounts. For measuring the 0.025 or 0.0125 c.c. quantities, a 0.2 or 0.25 c.c. pipet may be employed which is graduated either in 0.01 or 0.0125 c.c. amounts. If the graduations on these pipets are not well defined, it is well to mark off the desired measurements with a wax pencil.

1. *Preparation of Standard Antigen Suspension.*—Mix antigen with physiological salt solution according to required titer. Thus, if the titer is 1 c.c. antigen plus 1.1 c.c. salt solution. proceed as follows:

(a) Measure 1.1 c.c. salt solution into a standard antigen suspension vial.

(b) Measure 1 c.c. antigen into a similar vial.

(c) Pour the salt solution into the antigen, and as rapidly as possible (without waiting to drain the vial) pour the mixture back and forth 6 times to insure thorough mixing.

(d) Allow the antigen suspension to stand for 10 minutes before using. The suspension should not be used after 30 minutes standing.

One may mix more than 1 c.c. of antigen with a proportionately larger amount of salt solution, but not much less than 1 c.c. One c.c. when mixed with salt solution will be sufficient for about 15 tests: 2 c.c. of antigen mixed with salt solution will be sufficient for about 35 tests.

2. *Antigen Controls.*—After the antigen suspension has stood ten minutes. measure 0.025 c.c. into each of 3 tubes (controls) adding 0.15 c.c. saline to one, 0.15 c.c. negative serum to another and 0.15 c.c. positive serum to the third: shake for 3 minutes, add 0.5 c.c. saline to each and examine. The tubes containing positive

and negative serums are controls for the sensitiveness of that particular antigen suspension. The saline control is a gage of the opalescence of the suspension, and should contain no precipitate.

3. *Measuring Antigen Suspension.*—After the antigen control tests have been completed, shake the antigen suspension well (closing the mouth of the vial with the thumb) and measure 0.05, 0.025 and 0.0125 c.c. amounts for each serum, delivering the suspension to the bottom of the tubes. When employing the standard rack which contains 30 tubes, measure 0.05 c.c. amounts in the tubes of the first row; 0.025 c.c. amounts in the tubes of the second row and 0.0125 c.c. amounts in the tubes of the third row.

4. *Measuring Serum.*—The serum should be added as soon as possible after the antigen suspension has been pipeted, to avoid undue evaporation from the suspension. When examining large numbers of sera, it is well for one worker to measure the antigen suspension and for another to follow with the sera. Add 0.15 c.c. serum to each of the 0.05, 0.025 and 0.0125 c.c. amounts of antigen suspension, and shake the rack of tubes vigorously for about 10 seconds (by hand) to insure thorough mixing of the ingredients. The rack may now be set aside until a given number of tests—up to about 60—is ready for the regular 3-minute shaking period. When examining a small number of sera, it is well to permit the serum-antigen mixtures to stand for 10 minutes at room temperature before shaking for 3 minutes. This step will render more uniform the examination of small and large numbers of specimens.

5. *Shaking.*—The standard shaking period is 3 minutes. It is important not merely to agitate the rack of tubes but to see that the fluid within the tubes is vigorously agitated. When the tests are shaken by hand, one may shake each rack for 3 one-minute periods with short rest periods. When a shaking apparatus is employed, its speed should be not less than 275 oscillations and not more than 285 oscillations per minute, with a stroke of 1½ inch. When shaking by hand, this speed should be approximated.

6. *Addition of Salt Solution.*—After the serum-suspension mixtures have been shaken, add 1 c.c. salt solution to each tube of the first row of the rack (containing the 0.05 c.c. amounts of antigen suspension) and 0.5 c.c. salt solution to the remaining tubes. Shake sufficiently to mix ingredients.

7. *Reading of Results.*—Results may be read immediately after the addition of saline, but the final report should be based upon the findings after the tests have stood at room temperature 15 minutes after the addition of saline. Optimum reading conditions in each laboratory should be determined by trial. The following points will be found helpful:

(a) It is well to have but one source of light coming from a single window immediately in front of the observer. It will be found satisfactory to shade the upper and lower portions of the window, narrowing the source of light to a section several feet in height. Light from any other windows near the reader should be dimmed by lowering the window shades.

(b) When holding the rack in front of the exposed section of the window, the

definitely positive and the negative reactions are readily differentiated without lifting the tubes from the rack.

(c) In the case of weak reactions, examine each tube individually, lifting it several inches above the eye level and slanting it until the fluid is spread into a thin layer. The precipitate will then become readily visible.

(d) Those preferring magnification will find the substage mirror of the microscope helpful. Place mirror on reading table with concave surface upward. Hold the tube in slanting position 2 or 3 inches above the mirror and examine the image in the mirror. Either daylight or artificial light may be employed. One may also use an ordinary hand lens for reading the tests. A two- or three-fold magnification
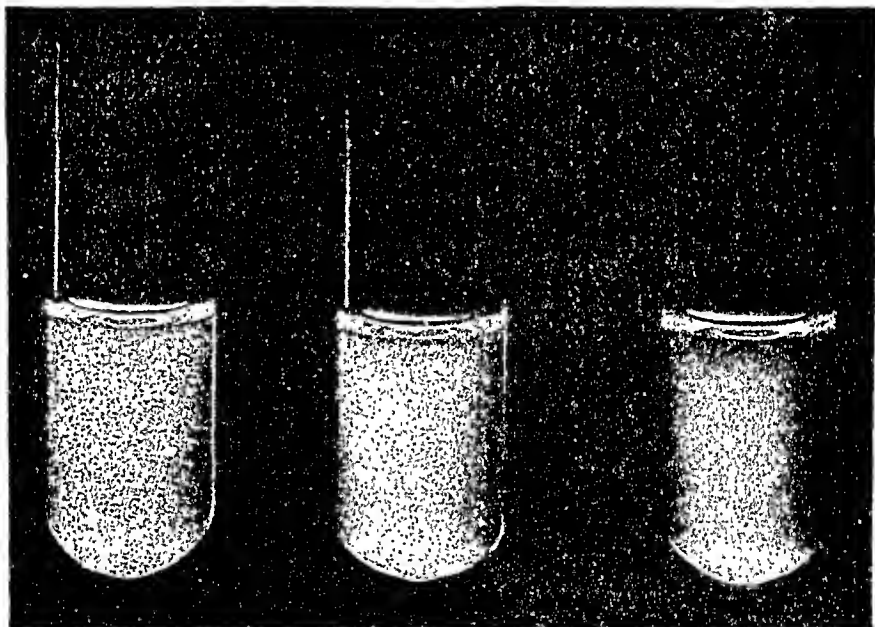
FIG. 341.—TYPES OF REACTIONS IN KAHN TEST
(From *Kahn Test*, Waverly Press, Inc., Baltimore)

will be found satisfactory. Some workers prefer the use of a slit-light arrangement, the source of light being an electric bulb enclosed in a box which is provided with a narrow slit.

As far as possible, workers should limit themselves to one method of reading. The occasional use of magnification by readers who usually do not resort to it will be likely to affect the uniformity of their reading scale. It should be emphasized that certain highly magnifying agglutinoscopes show particles in serum alone, and are thus unfit for use in the test. The magnification must be sufficiently low as to assure opalescent and clear-cut negative reactions, with entire freedom from visible particles.

8. *Types of Reactions.*—The reactions are read on a plus-sign basis (Fig. 341).

(a) Four-plus reactions. In these reactions, definitely visible particles are sus-

pended in a transparent or opalescent medium. The individual particles are readily visible by direct examination, without lifting the tubes from the rack.

(b) *Three-plus reactions.* In these reactions, the particles are also definitely visible, but are less clear-cut than in four-plus reactions. The particles are not always distinguishable until the tube is lifted from the rack and examined individually.

(c) *Two-plus reactions.* In these reactions, finer particles are suspended frequently in a somewhat turbid medium. The particles cannot be distinguished until the tube is examined individually, usually by slanting.

(d) *One-plus reactions.* In these, still finer particles are suspended in a somewhat turbid medium.

(e) *Doubtful reactions.* In these, extremely fine particles, just within the visible range, are suspended in a somewhat turbid medium.

(f) *Negative reactions.* In these, the medium is transparent and opalescent and free from visible particles. In the rack, negative reactions are readily distinguished from weakly positive reactions by the fact that the latter appear turbid.

The American Committee on Evaluation of Serodiagnostic Tests for Syphilis recommends that reports be rendered as positive, doubtful or negative.

9. *Interpretation of Results.*—The results in the individual tubes are read on a plus-sign basis. The interpretation of the results is also on this basis. When each of the three tubes shows four-plus precipitation, the final result is interpreted as a four-plus reaction. When some of the tubes show weak or negative precipitation, such as negative in the first tube, two-plus in the second tube, and four-plus in the third tube, the final result is interpreted as a two-plus reaction. In other words, the final result is the average of the findings of the number of plus signs represented by the three tubes? If, after averaging, the final result contains a fraction of one-plus, then, if the fraction is one-third, it is disregarded; if the fraction is two-thirds, it is counted as an additional plus sign. Examples: ++, +++++, +++++, is interpreted as a three-plus reaction, and +, +++++, +++++, is also interpreted as a three-plus reaction. When precipitation in an individual tube is read doubtful (±), it is not taken into consideration in averaging, but is counted as negative. Thus, ±, +, ++++ is interpreted as a one-plus reaction (four-plus divided by three equals one-plus). For reasons of conservatism, very weak reactions, *i.e.*, those in which precipitation is limited to the third tube, no result is averaged unless this tube shows at least a two-plus precipitate. Thus —, —, ++ is interpreted as a doubtful (±) reaction. When the results in the three tubes are —, ±, +, the reaction is interpreted as negative. It might be added, however, that even the latter reactions have been found to be highly specific for syphilis. The table reproduced on the following page gives an adequate outline of the test.

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[1] In rare instances, a reaction is obtained in which precipitation is marked in the first tube and weak or negative in the second and third tubes. In such a case it may be found that a 2:1 or 1:1 ratio of serum to antigen suspension may give strong precipitation reaction. Then the serum reaction is considered as ++++.

	Tube 1	Tube 2	Tube 3	Completion of Test
Serum: antigen suspension	3:1	6:1	12:1	Tests are shaken 3 minutes, 1 c.c. salt solution is added to first tube and 0.5 c.c. to other tubes and results are read.
Antigen suspension, c.c...	0.05	0.025	0.0125	
Serum (heated at 56° C. for 30 minutes), c.c...	0.15	0.15	0.15	

10. *Recording of Results.*—A permanent record of the findings in all tubes of each test should be made at the time of reading. Preferably the tests should be read independently by two workers.

11. *Serum Controls.*—Examine each serum for particles which might give the appearance of a specific precipitate. It is essential to determine, particularly in the case of positive reactions, that the serum used in the test is entirely clear. In such reactions it is well to dilute the serum with salt solution to correspond approximately to the final dilution of serum when the test is completed. This dilution of the serum may render visible fine particles which are invisible in undiluted serum.

12. *Procedure with Less Than Three Tubes.*—If there is insufficient serum for the regular three-tube test, examine and report as follows:

(*a*) If enough serum for two tubes, employ the lesser amounts of antigen suspension; report as a two-tube test.

(*b*) If enough for one tube, employ the least amount of antigen suspension; report as a one-tube test.

(*c*) If less than 0.15 c.c. serum is available, a one-tube test (micro) may be made by employing 10 parts of serum to 1 part of antigen suspension. Thus, if 0.05 c.c. serum is available, it is employed with 0.005 c.c. antigen suspension. Report these reactions as micro tests.

Temperature for Performing Kahn Test.—It is essential that the temperature of the room during the performance of the Kahn test be close to 21° C.

Quantitative Procedure.—The standard test is only partially quantitative. A quantitative relationship exists between sera giving $+ + + +$, $+ + +$, $+ +$, $+$ or $\pm$, but two sera giving $+ + + +$ reactions might show marked variation in potency. The degree of this variation may readily be determined by means of the quantitative procedure. Only positive sera are employed. The test consists of two steps. The sera are first diluted in series with salt solution, then each dilution is tested with antigen suspension, and the highest dilution giving a positive precipitation reaction is the end point desired.

Many workers use the more sensitive "sensitized" antigen in preference to standard antigen in the quantitative tests with serum and spinal fluid. Since these tests are made only in cases in which syphilis is definitely established, it is believed that the use of a highly sensitive method is more desirable than the use of a conservative method.

1. *Serum Dilutions.*—Prepare a series of 8 serum dilutions with physiological salt solution, so that the ratio of the volume of diluted serum to the volume of

serum before dilution ranges from 1 (undiluted serum) to 60 (1 part serum plus 59 parts salt solution). The following scheme is employed:

Dilution Number	Dilution Ratio		
(1)	1	=	undiluted serum
(2)	5	=	0.2 c.c. undiluted serum plus 0.8 c.c. salt solution
(3)	10	=	0.7 c.c. of (2) plus 0.7 c.c. salt solution
(4)	20	=	0.2 c.c. of (3) plus 0.2 c.c. salt solution
(5)	30	=	0.2 c.c. of (3) plus 0.4 c.c. salt solution
(6)	40	=	0.1 c.c. of (3) plus 0.3 c.c. salt solution
(7)	50	=	0.1 c.c. of (3) plus 0.4 c.c. salt solution
(8)	60	=	0.1 c.c. of (3) plus 0.5 c.c. salt solution

2. *Antigen Suspension.*—Prepare standard antigen suspension as for regular 3-tube test (or sensitized antigen as described under Presumptive Procedure). Measure 0.01 c.c. amounts of antigen suspension into 8 standard test tubes.

3. *Measuring Serum Dilutions.*—Add 0.15 c.c. amounts of the 8 serum dilutions, in order, beginning with the highest dilution (8), to the tubes containing antigen suspension. Mix ingredients by shaking for about 10 seconds.

4. *Shaking.*—Shake the mixtures of serum and antigen suspension by hand or in shaking apparatus for three minutes at 275 to 285 oscillations per minute.

5. *Addition of Saline.*—Add 0.5 c.c. salt solution to each tube and shake by hand for a few seconds to mix ingredients.

6. *Reading Results.*—After the salt solution has been added, the results are read. Record a definite precipitate ($+ + + +$, $+ + +$ or $+ +$) as positive and a weak or negative precipitate ($+$, $\pm$ or $-$) as negative.

7. *Determination of Reacting (Kahn) Units.*—If a serum gives a positive reaction only before dilution, it is considered as containing 4 units. The potency of any serum is determined according to the formula $S = 4D$, where S is the serum potency in terms of reacting units and D is the highest dilution ratio giving a positive reaction.

8. *Highly Potent Sera.*—If a serum gives a positive precipitation reaction with a dilution ratio of 60, examine still higher dilutions of serum with antigen suspension until a positive reaction is no longer obtained. Higher dilutions may be readily prepared by resorting to dilution (3), of which an excess is prepared. Thus, continuing the dilution numbers of the above outline, we would have:

Dilution Number	Dilution Ratio		
(9)	70	=	0.05 c.c. of (3) plus 0.3 c.c. salt solution
(10)	80	=	0.05 c.c. of (3) plus 0.35 c.c. salt solution
(11)	90	=	0.05 c.c. of (3) plus 0.4 c.c. salt solution
(12)	100	=	0.05 c.c. of (3) plus 0.45 c.c. salt solution

If necessary, still higher dilution ratios may be prepared, until a negative reaction is obtained.

The Presumptive Procedure.—The presumptive procedure is a one-tube test and is more sensitive than the regular Kahn test by virtue of the fact that it utilizes

a highly sensitive antigen known as "sensitized antigen." As is true in the case of standard Kahn antigen, sensitized antigen also possesses a uniform degree of sensitiveness. In preparing this antigen, standard antigen is used as a base and is brought to the required sensitiveness of sensitized antigen by means of sensitizing reagent in combination with cholesterolized alcohol. Experience has shown that the addition of 1 to 25% sensitizing reagent to standard antigen followed by the addition of 10 or 25% cholesterolized alcohol will considerably increase the sensitiveness of standard antigen. This fact is generally utilized in the standardization of sensitized antigen.

Preparation of Sensitized Antigen.—In preparing sensitized antigen using standard antigen as a base, the following steps are employed:

1. To 10 c.c. of standard antigen are added 0.1 c.c. of sensitizing reagent and 1 c.c. cholesterolized alcohol (1% sensitizing reagent plus 10% dilution with cholesterolized alcohol).

2. To a second 10 c.c. amount of standard antigen are added 0.2 c.c. of the sensitizing reagent and 1 c.c. cholesterolized alcohol (2% sensitizing reagent plus 10% dilution with cholesterolized alcohol).

Titration of Antigen.—These two modified antigens are titrated in the usual manner to determine the smallest amount of salt solution to add to 1 c.c. of antigen resulting in an antigen suspension. the aggregates of which will completely disperse in additional salt solution or in nonsyphilitic serum. In making this titration, antigen suspensions are prepared by mixing 1 c.c. amounts of antigen with 1.7, 1.9, 2.0, 2.1 and 2.2 c.c. quantities of salt solution, respectively. After these suspensions have stood for 30 minutes at room temperature, they are shaken and examined for the dispersability of the aggregates as follows: Each of the antigen suspensions is tested by employing the regular 3-tube test except that after depositing the 0.05. 0.025 and 0.0125 amounts of the suspension in 3 tubes, 0.15 c.c. amounts of physiological salt solution instead of serum are added to each tube. After the usual 3-minute shaking period, 1 c.c. salt solution is added to the tube containing the 0.05 c.c. amount of antigen suspension and 0.5 c.c. amounts of salt solution to the remaining tubes. The antigen suspension containing the smallest amount of salt solution and having aggregates which are completely dispersable in the additional salt solution. represents the titer of each of the modified antigens.

Determination of Sensitiveness of Antigen.—1. The 2 modified antigens at their titers are then compared in sensitiveness with a known standard sensitized antigen employing 9 weakly positive sera and 1 negative serum. In these comparative examinations the regular 3-tube test is employed. If one of the modified antigens is comparable in sensitiveness to the sensitized antigen, the comparative examination is repeated with 20 additional weakly positive and 20 negative sera, and if the results are again comparable, the new antigen is considered as standard sensitized antigen.

2. If neither one of the 2 modified antigens conforms to the requirements of standard sensitized antigen, other combinations of sensitizing reagent and cholesterolized alcohol are tried.

When the correct sensitiveness has been obtained with the sample of antigen, any desired amount may be prepared, adhering to the proportions experimentally established. The new antigen should then be compared with standard sensitized antigen, using at least 40 sera. If the comparison is favorable, the new antigen may be considered ready for use.

After the desired potency of sensitized antigen has been obtained, any amount of the antigen can be prepared by adding to standard antigen the determined amounts of sensitizing reagent plus cholesterolized alcohol.

Performance.—1. Pipet 1 c.c. of standard sensitized antigen into an antigen suspension vial.

2. Pipet an amount of physiological salt solution, indicated by the titer, into a similar vial.

3. Pour the salt solution into the antigen and, as rapidly as possible, pour the mixture back and forth 6 times.

4. Allow the antigen suspension to stand 10 minutes at room temperature before using.

5. Prepare antigen controls as with standard antigen.

6. Measure 0.025 c.c. of the thoroughly mixed antigen suspension into a standard tube (7.5 centimeters in length, 1 centimeter in diameter) with a 0.25 c.c. pipet marked in 0.025 c.c. amounts or with a 0.2 c.c. pipet marked in 0.001 c.c., delivering to the bottom of the tube.

7. Add 0.15 c.c. serum, after heating for 30 minutes at 56° C., with a 1 c.c. pipet graduated in 0.01 c.c. and mix the serum with the antigen suspension by shaking the rack vigorously by hand for about 10 seconds.

8. Shake rack in the usual manner for 3 minutes (oscillation speed 275 to 285 per minute).

9. Add 0.5 c.c. physiological salt solution to the tube and examine for presence of precipitates.

10. The results are interpreted on a qualitative basis. Marked precipitation reactions (+ + + +) are interpreted as positive; moderate precipitation reactions (+ + +, + +) are interpreted as weakly positive, while very weak reactions (+, ±) are classed as negatives.

11. Prepare serum controls as in the standard test.

Value of Presumptive Procedure.—According to Kahn, the following are the most important purposes of this procedure:

1. As a technical check on the regular Kahn test. All sera giving positive reactions in the regular test should be positive with the presumptive procedure. A positive Kahn and negative presumptive indicate an error in technic.

2. As a check on weak Kahn reactions. A serum giving a doubtful (±) Kahn reaction should give a positive presumptive reaction; if negative, the doubtful reaction is most likely due to particles present in the serum.

3. As an aid in the diagnosis in certain cases of syphilis in which the regular Kahn test is negative.

4. Due to its high sensitiveness, the presumptive procedure, when negative, is a

greater criterion for establishing absence of syphilis than the regular Kahn test.

Standard (Diagnostic) Test with Spinal Fluid.—In this test, the greater part of the spinal fluid globulins is precipitated by means of ammonium sulphate and redissolved in an amount of physiological salt solution equivalent to a tenth of the original spinal fluid volume. The concentrated globulin solution thus obtained is then tested with antigen suspension.

1. Preparation of Concentrated Globulin Solution.—The reagents needed for the preparation of concentrated globulin solution are: (a) spinal fluid, (b) physiological salt solution and (c) a solution of saturated ammonium sulphate. This salt must be of highest purity (Baker's Analyzed or Merck's Reagent).

Procedure.—(a) Centrifuge spinal fluid to render it free from cells and foreign particles.

(b) Add 1.5 c.c. of the clear fluid to a standard Kahn test tube (7.5 by 1 cm.).

(c) To the same tube add 1.5 c.c. of a saturated solution of ammonium sulphate.

(d) Mix fluids, covering mouth of tube with thumb (protected with rubber if desired) and shake tube back and forth vigorously. The thorough mixing of the spinal fluid and ammonium sulphate is of great importance. Place mixture in 56° C. water bath for 15 minutes to hasten the precipitation of the globulins.

(e) Centrifuge mixture at high speed for about 15 minutes to completely throw down the precipitated globulins.

(f) Remove the supernatant fluid *as completely as possible.* This is best accomplished with the aid of a finely drawn capillary pipet. The major amount of supernatant fluid is first withdrawn. The tube is then slanted at an angle of about 45 degrees and the remaining fluid is withdrawn after bringing the opening of the capillary pipet to the point of contact of the globulin precipitate and the inner wall of the tube. It will be found that the last trace of supernatant fluid can be removed by this method. Some workers prefer to pour off the supernatant fluid and place the inverted tube in a rack having a layer of filter paper on the bottom. It will be found that after about 10 minutes standing, the fluid in the tube will be completely drained and absorbed by the filter paper.

(g) Add 0.15 c.c. salt solution to the precipitate and redissolve it by gentle shaking. In adding this salt solution the point of the pipet should be lowered close to the bottom of the tube to avoid washing down the ammonium sulphate adhering to the inner wall. The globulin precipitate will dissolve readily. This globulin solution is now ready to be tested with antigen suspension.

2. Preparation of Antigen Suspension.—Mix salt solution with antigen in the same manner as for the test with serum, according to the antigen titer required for spinal fluid. The antigen suspension should stand 10 minutes before its use in the test and should be used within 30 minutes. Control tests of the antigen suspension should be made, as outlined under "Performance of the Standard Test" for serum.

3. Measuring of Antigen Suspension.—With a 0.2 c.c. pipet graduated to 0.001 c.c., measure 0.01 c.c. of antigen suspension to the bottom of a standard Kahn test tube.

1. Measuring of Concentrated Globulin Solution.—Measure 0.15 c.c. of concentrated solution into the antigen suspension tube, using a 0.2 c.c. pipet. Shake tests vigorously for 10 seconds to mix ingredients.

5. Controls.—Include positive and negative spinal fluid controls; also observe each concentrated globulin solution to establish that it is free from foreign particles.

6. Shaking.—After mixing the concentrated fluid with antigen suspension, shake test at standard speed for 4 minutes. This period is more desirable for spinal fluids than 3 minutes.

7. Addition of Salt Solution.—Add 0.5 c.c. physiological salt solution to tube.

8. Reading of Results.—A definite precipitate suspended in a clear medium is read $+ + + +$. Proportionately weaker precipitates are read $+ + +$, $+ +$ and $+$ respectively.

In practice the spinal fluid test herein described should be carried out in duplicate.

Presumptive Test with Spinal Fluid.—This test is carried out essentially as the standard test with spinal fluid, except that sensitized antigen is used.

Quantitative Test with Spinal Fluid.—Employing spinal fluids that are known to give positive reactions, a series of dilutions are made similar to those of positive sera. The dilution range is from undiluted to 1:10. Undiluted spinal fluid is considered as equivalent to a 1:10 dilution, since the standard test is performed with a solution in which the globulin is concentrated to one-tenth the original volume. In the performance of the test, 0.01 c.c. antigen suspension is mixed with 0.15 c.c. of each of the spinal fluid dilutions. The mixtures are shaken for 3 minutes, 0.5 c.c. salt solution is added to each tube and the results are read on a similar basis to the quantitative serum tests.

Miscellaneous Tests.—*Local Reaction.*—The Kahn test may be used in determining the serologic reactions of fluid obtained from chancres and other syphilitic lesions. After cleansing with physiological salt solution, and under moderate pressure, fluid from chancres and other lesions is collected by means of a fine capillary pipet. This is deposited at the bottom of a small agglutination tube and centrifuged to throw down cellular matter. The clear supernatant fluid is mixed with standard antigen suspension in the proportion of 10:1. If 0.03 c.c. of fluid is available, it is mixed with 0.003 c.c. of antigen suspension. The test is shaken for 3 minutes and 0.2 c.c. salt solution added before reading the results. A definite precipitate is read positive, while freedom from precipitate is read negative.

Aqueous Humor Reaction.—Fralick recently reported a series of Kahn reactions with aqueous humor. The fluid is treated similarly to spinal fluid by concentrating the globulins before performing the test.

KLINE MICROSCOPIC TEST FOR SYPHILIS [4]

Sera.—These are prepared as for the Wassermann test, care being exercised that they contain no red blood cells, or foreign particles. (They are heated at 56° C. for 30 minutes.)

[4] B. S. Kline, *Am. Soc. Clin. Lab. Tech.*, 1934, 1:4.

When blood is obtainable in small quantity only it is advisable to collect this in a narrow test tube (about 8 to 9 mm.) and to handle it in the same manner as a larger sample from the vein.

When blood is obtainable in very small quantity only it is advisable to collect this in a narrow glass tube with a capillary end. The end is then sealed, a narrow rod is passed through the open end to free the clot from the wall and after the tube is centrifuged at high speed, it is placed in a water bath at 56° C. with water above the upper level of the serum. After inactivation, the tube is filed and broken just above the clot and the serum allowed to run into or is drawn into a 1 c.c. pipet, graduated in hundredths.

Glassware.—Microscopic slides 2×3 inches as purchased are rubbed on both sides with Bon Ami paste (prepared by breaking up a cake of Bon Ami in a small quantity of hot water). As soon as the paste is dry (in about 5 minutes) it is completely removed from the slide with a soft muslin cloth. For convenience the slides covered with paste may be stuck to each other, allowed to dry, and cleaned at any time.

Upon clean slides for the heated serum tests, 12 paraffin rings, each with an inside diameter of 14 mm. are mounted. Inasmuch as the slide test results are influenced by the surface area of the chambers it is important that the paraffin rings be thin ones. With a little practice the required amount of paraffin can be ascertained. At first. it may be difficult to make complete rings. These incomplete rings may be completed by applying the loop a second time to the open areas.

For the spinal fluid tests double ring slides are prepared as follows: Upon the clean slide a steel mold 3 2/16×2 3/16×⅛ inches with 2 central walls 1 0/16 inches in diameter is placed. A metal disk 1 5/16 inches in diameter and 3/16 of an inch thick is then placed in the center of each well. The space between them is filled with hot wax (2 parts ordinary vaseline and 1 part parowax) from a 10 c.c. glass syringe. After the mixture cools a few minutes. each disk is elevated from the slide and separated from the wax wall by turning the central screw handle a few times to the right (holding mold down at edge). After the disk is freed, it is lifted out. The mold is removed by inserting a thin blade between it and the slide.

Pipets.—The pipets needed for delivering sera, and spinal fluid, and those for preparing the antigen emulsions are the ordinary finely graduated 0.2 to 10 c.c. pipets. The pipet for the 1% acetic acid is a 0.2 c.c. pipet graduated in 0.001 c.c. The pipets for delivering the antigen emulsions are Wright pipets made from glass tubing 6 to 10 mm. in diameter with the tubes about 0.5 mm. in outside diameter, delivering a drop equal to about 0.008 c.c. (62 drops per 0.5 c.c.).

Instrument for Making Paraffin Rings.—This is essentially the instrument proposed by Green. A piece of soft iron wire (No. 28) is wound twice tightly about a test tube (about 15 mm. in outside diameter) forming a double loop and leaving a double shaft about an inch in length. The two shafts are then twisted together to within a quarter of an inch of the free end. After removing the looped wire from the test tube, a piece of linen thread (No. 12) is started from the free end of the shaft after being fastened here by a single twist of the free ends. Three long turns

are made reaching the loop which is then tightly wound with the thread. The winding is continued up the shaft to the free end where it is fastened between the two ends of the wire by twisting them. The loop is then bent at right angles to the shaft. It is then reshaped by working the loop against the bottom of the test tube mentioned above. The shaft is then inserted into the handle of a teasing needle or into a straight hemostatic forceps.

The paraffin rings are made by dipping the instrument into smoking paraffin (about 120° C.) draining quickly at one point and transferring the remainder to the glass slide.

Slide Holders.—The slide holder (for 3×2 inch slides) is a wooden lid of a slide box (3½×6¾×½ inches) containing an easily fitting thin wooden shelf having a small handle at each end.

Salt Solution.—0.85% sodium chloride (C.P. or reagent, Merck) solution used in the tests is prepared with distilled water having a pH of about 6. (Such water gives a lilac color when one drop of chlorphenol red indicator (LaMotte) is added to 0.25 c.c. of it in a small chamber). Distilled water having a pH of 5.2 or less gives a yellow color with this indicator and is not satisfactory.

1% Acetic Acid.—(C.P. Reagent.)—It is advisable to use no less than 1 c.c. of acid (delivered from a 1 or 2 c.c. pipet) and accordingly 99 c.c. of distilled water.

Antigen.[5]—The purified antigen used in the microscopic slide precipitation tests for syphilis is prepared as follows:

1. Two-hundred grams of dried heart powder (Difco) is placed in a 2 liter Erlenmeyer flask.

2. One liter of absolute ethyl alcohol (99 + %) (Rossville Commercial Alcohol Corp., Lawrenceville, Ind.) is added.

3. After the flask is stoppered with a cork covered with tin foil, it is shaken vigorously by hand at intervals for 2 hours. Better still 2 wide mouth bottles (Difco bottles for 1 pound beef heart powder) each with 100 grams of beef heart powder and 500 c.c. of absolute ethyl alcohol (99 + %) are shaken vigorously in a machine for 2 hours. (This short extraction removes almost all of the desired antigenic substance in the powder.)

4. The extract is filtered into a liter cylinder through good grade filter paper of medium texture (Schleicher and Schull No. 597; 38.5 cm.).

5. During filtration the mixture is stirred with a wooden tongue depressor and toward the end pressed with the cork until the powder is quite dry.

6. The extract (about 775 c.c.) is placed in the refrigerator at 8° to 10° C. for 24 hours.

During this time a fairly heavy white precipitate settles out. This is filtered off

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[5] Standard materials including antigen for the microscopic slide precipitation tests for syphilis may be obtained from the LaMotte Chemical Products Company, McCormick Building, Baltimore, Md.

Magath has described a machine for holding and tilting the slides and other apparatus (*J. Lab. & Clin M.*, 1932, 18.83).

and the filtrate in a large evaporating dish is concentrated on a water bath at 45° to 50° C. determined by a thermometer bulb within the extract. During evaporation of the alcoholic extract an irregular festoon appears at the periphery. When the extract reaches the proper concentration the festoon disappears and the margin of the concentrated extract is sharp.

7. The extract is now poured quickly into 500 c.c. of acetone, C.P. (Coleman and Bell) at 50° C. in a large evaporating dish.

8. The dish is then placed in an air incubator at 37° C. for 15 minutes after which the acetone is decanted leaving a soft yellow brown wax adherent to the side of the dish. (Longer periods of precipitation and precipitation at lower temperatures permit of precipitation of adventitious substances as well and such antigens give more sensitive and less specific results.

9. The dish is then placed on a water bath or in an air incubator at 50° C. until the little acetone remaining has evaporated (about 30 minutes).

10. The wax is then worked together and placed in a glass-stoppered bottle. Then 80 c.c. of absolute ethyl alcohol (99 + %) that has been kept in an air incubator at 50° to 56° C. for one-half hour or longer, is added and after a few minutes' shaking the bottle is placed in an air incubator at 50° C. and shaken gently after 15 minutes and again after 30 minutes. when it is removed from the incubator and placed in the refrigerator at 8° to 10° C. for 45 minutes.

11. The solution is then filtered and the filtrate is evaporated down at 45° to 50° C. resulting in a soft brown wax (antigen wax). The wax is weighed and to each gram in a glass-stoppered bottle. 10 c.c. of absolute ethyl alcohol (99 + %) (at 50° to 56° C.) is added. After the bottle is shaken for a few minutes it is placed in an air incubator at 50° for 30 minutes, and then shaken a few minutes.

12. The slightly turbid solution is then placed at 8° to 10° C. for about an hour and then filtered. The resultant clear filtrate is the antigen, and contains about 8.75% of the alcohol-treated acetone-insoluble wax.

The average yield of antigen wax from ½ pound of beef heart powder is 3.5 to 4 grams. The discarded acetone-soluble lipoidal residue (impurities) ordinarily weighs at least 3 times this amount.

The antigen keeps best at room temperature hermetically sealed in glass containers. In small-necked glass-stoppered bottles (opened from time to time) kept at room temperature it shows no appreciable change in specificity or sensitivity for at least 6 months. (As stated above it is important to follow the steps outlined in minute detail. Too long a precipitation in acetone, precipitation at too low a temperature and use of improper chemicals for instance result in waxes which contain some impurities that determine a sensitivity greater than standard.)

Preparation of Antigen Emulsions for Diagnostic and Exclusion Slide Tests of Heated Serum.—Formula.—0.85 c.c. of distilled water (pH about 6).

1.0 c.c. 1% cholesterin (C.P. Pfanstichl) in absolute ethyl alcohol (99 + %).

0.1 c.c. antigen.

2.45 c.c. 0.85% sodium chloride (C.P. or reagent, Merck) solution (pH about 6). The 1% cholesterin solution for the emulsions is prepared in about 45 minutes

by placing the cholesterin flakes and absolute alcohol in a glass-stoppered bottle in an oven at 50° to 56° C. and shaking gently a few minutes at 15-minute intervals. The solution kept in the incubator at 37° C. is thoroughly satisfactory for use as long as 2 months.

The sensitivity of an emulsion is greatly influenced by the quantity of cholesterin present. The sensitivity is likewise influenced by the quality of the cholesterin used. The cholesterin (Pfanstiehl, C.P.) that has been found uniformly satisfactory in the slide tests is flaky, pearly and readily soluble to 1% in absolute ethyl alcohol (99 + %). Powdery, white cholesterin incompletely soluble to 1% in absolute alcohol (99 + %) has been found to give too sensitive results.

The technic of preparing the emulsion according to the above formula is as follows: Into a 1 ounce bottle the required amount of distilled water (pH about 6) is pipetted.

The bottle is held at an angle, and the 1% cholesterin in absolute ethyl alcohol (99 + %) is allowed to run along the side of the neck of the bottle.

The bottle is gently rotated from the neck for 20 seconds.

It is held at an angle again, and the proper amount of antigen is pipetted against the side of the neck of the bottle from a finely graduated pipet.

The bottle is promptly stoppered with a cork and shaken vigorously (the fluid thrown from bottle to cork and back) for 1 minute.

Lastly, the 0.85% sodium chloride solution is allowed to run in quite rapidly, the bottle is stoppered again and shaken less vigorously than previously for 1 minute.

The emulsion, when examined under the microscope at a magnification of about 120 times, shows numerous very fine particles, but no clumps whatever.

For Diagnostic Test

Place 1 c.c. or more of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 35° C. (beaker of water in usual laboratory air incubator at about 37° C.) for 15 minutes. The emulsion as soon as heated is ready for use.

For Exclusion Test

Place 2 c.c. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 56° C. for 15 minutes. Then pour into a 3×1 inch tube and centrifuge for 15 minutes (eighth setting Rheostat, Centrifuge Size 1, Type SB). Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1.5 c.c. of 0.85% sodium chloride solution. Transfer to a narrow tube for use.

These emulsions, kept at room temperature, are satisfactory for use for forty-eight hours after preparation.

Diagnostic and Exclusion Microscopic Slide Precipitation Tests for Syphilis with Heated Serum.—1. Place 3 heated serum test slides each with 12 small chambers, on a tray in a small holder.

Into each of the 36 rings, pipet 0.05 c.c. of the heated serum to be tested era in duplicate).

After all the sera are pipetted, 1 drop of the diagnostic test antigen emulsion t 0.008 c.c.) is allowed to fall from a Wright pipet into one of the two por of each serum. Into each of the other 18 duplicate sera a similar drop of ion test antigen emulsion is allowed to fall from a Wright pipet.
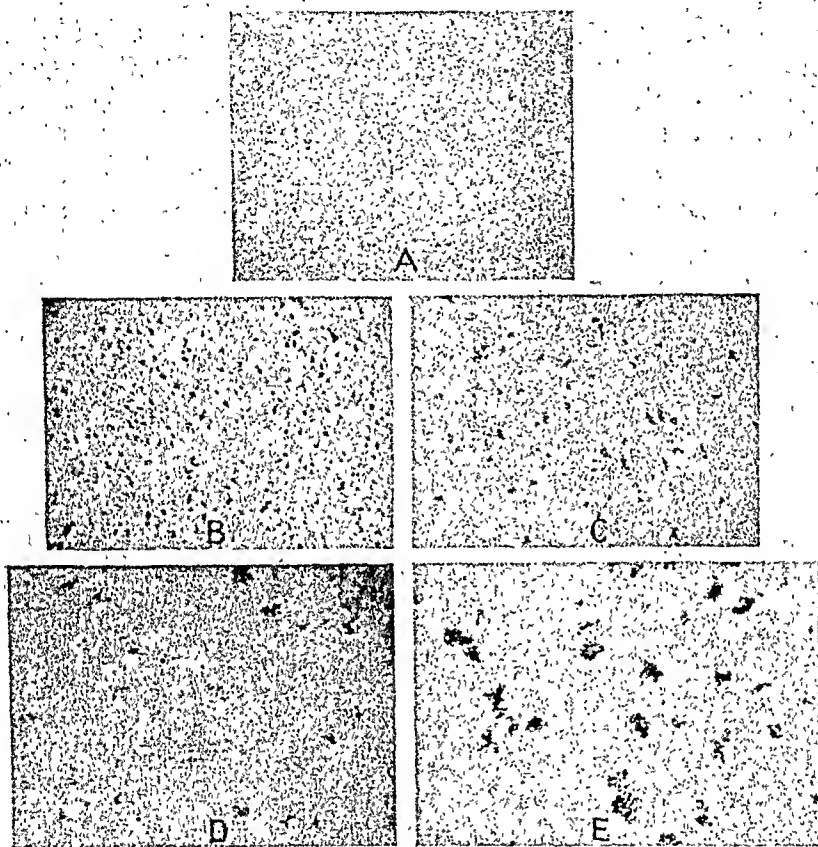
FIG. 342.—THE KLINE MICROSCOPIC PRECIPITATION REACTION.

negative reaction; B, positive (+); C, positive (++); D, positive, (+++); E, ve (++++).

The slides in the holder are rotated on a flat surface for 4 minutes.

The results are examined at once through the microscope at a magnification of t 120 times (low power 16 mm. objective, eyepiece 12) with the light cut as for the study of urinary sediments and reported in terms of pluses rding to the degree of clumping and the size of the clumps (Fig. 342). y spilling from the chamber makes the reaction therein unsatisfactory, and erum concerned should be retested.

sufficient serum is available the exclusion test for syphilis may be done with .c. heated serum in a chamber similar to that employed for the spinal fluid test mm. in diameter) and one drop about 0.008 c.c. of emulsion made by

suspending the sediment of 8 c.c. of exclusion test emulsion (centrifuged 15 minutes at 8th rheostat setting) in 1 c.c. of 0.85% salt solution.

Preparation of Antigen Emulsions for Diagnostic and Exclusion Slide Tests of Spinal Fluid.—Formula.— 0.85 c.c. distilled water (pH about 6).

1.25 c.c. of 1% cholesterin (Pfanstiehl C.P.) in absolute ethyl alcohol (99 + %).

0.1 c.c. antigen.

2.2 c.c. of 0.85% sodium chloride (C.P. or reagent, Merck) solution (pH about 6).

8.8 c.c. of the emulsion are made by using double the quantities given in the formula.

For Diagnostic Test	For Exclusion Test

For Diagnostic Test

Place 4 c.c. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 35° C. for 15 minutes. Then pour into a 3×1 inch tube. Centrifuge for 15 minutes (eighth setting Rheostat, size 1, S.B.). Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1 c.c. of 0.85% sodium chloride solution (pH about 6).

Transfer to a narrow test tube for use.

For Exclusion Test

Place 4 c.c. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 50° C. for 15 minutes. Then pour into a 3×1 inch tube. Centrifuge for fifteen minutes (eighth setting Rheostat, size 1, S.B.). Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1 c.c. of 0.85% sodium chloride solution (pH about 6).

Transfer to a narrow test tube for use.

These emulsions, kept at room temperature, are satisfactory for use for 24 hours.

Diagnostic and Exclusion Microscopic Slide Precipitation Tests for Syphilis with Spinal Fluid.—Preliminary Procedures.— Spinal fluids, turbid with exudate, blood or bacteria, or containing injected substances including horse serum, are unsatisfactory for testing. Spinal fluids with slight turbidity or few particles are centrifuged at high speed for 10 minutes, and the clear fluid is withdrawn or decanted.

Place the required number of test tubes, 6×⅝ inches, each containing 5 c.c. of Benedict's solution (1909-1910), in a beaker (Pyrex). Add water halfway to the top. Heat. Keep the tubes in vigorously boiling water for 5 minutes.

Place the tubes in a rack. After making certain that no copper reduction has occurred in any of the tubes, add to each tube, properly numbered, 0.5 c.c. of spinal fluid. Shake each tube vigorously for 10 seconds.

Replace the tubes in the beaker. Add water halfway to the top. Heat. Keep the tubes in vigorously boiling water for 5 minutes.

Replace the tubes in the rack, inspecting each immediately after removal from the beaker, for precipitate indicating presence of sugar.

Spinal fluids giving a negative reaction for sugar in the above test are unsatis-

factory for testing for syphilis. These are fluids that have been acted upon by bacteria either inside or outside of the body. In the former case (bacterial meningitis) in which organisms and ferments of the exudate have acted upon the sugar, the fluids may contain substances that give positive or unsatisfactory reactions in various tests for syphilis. Bacterial contamination subsequent to withdrawal of spinal fluids from cases of syphilis causes a steady loss both of sugar and of the specific reacting substance, if the fluids are kept at room temperature.

On the other hand, if spinal fluids containing sugar, when withdrawn from the body are kept at low temperature (8° to 10° C.), they continue to give a positive reaction for sugar with the test described above for several weeks, and syphilitic spinal fluids under these conditions show no appreciable loss of specific reacting substance for at least a week.

The clear and cleared spinal fluids which give a positive reaction in the sugar test described above are then tested as follows:

1. Into each of 12 chambers (33 mm. in diameter) on 6 glass slides (in a holder tilted slightly by placing a small metal bar ⅛ inch thick under one long slide) deliver 0.05 c.c. of 1% glacial acetic acid solution from a 0.2 c.c. pipet graduated in thousandths. (It is of the utmost importance that the 1% acetic acid be carefully prepared since this reagent in stronger concentration will precipitate the emulsion.)

2. Into each chamber allow 0.25 c.c. of the spinal fluid to be tested (6 spinal fluids in duplicate) to fall from a 1 c.c. pipet graduated in hundredths. Hold the pipet directly above the acid and lastly touch the tip of the pipet at some dry portion of the chamber.

3. Rotate the slides in the holder on a flat surface with moderate vigor for 1 minute.

4. Into one-half of the chambers allow one drop (about 0.008 c.c.) of diagnostic antigen emulsion to fall from a Wright pipet.

5. Into each of the duplicate spinal fluids 1 drop (about 0.008 c.c. of exclusion antigen emulsion) is allowed to fall from a Wright pipet.

6. Rotate the slides in the holder on a flat surface with moderate vigor for 1 minute to distribute the antigen and then for 4 minutes move the holder gently but rapidly (about three complete movements a second) back and forth a distance of ¼ to ½ inch.

7. The results are examined at once through the microscope at a magnification of about 120 times (objective 16 mm. eyepiece 12) with the light cut down as for the study of urinary sediments and recorded in terms of pluses according to the degree of clumping and the size of the clumps. For ease in reading the results, the slide is tilted on a piece of metal ⅛ inch thick, ½ inch wide, and 4 inches long, placed on the stage.

Readings.—The results are read at once through the microscope at a magnification of about 120 times (low power 16 mm. objective, eyepiece 12) with the light cut down as for the study of urinary sediments. The results are reported according to the degree of clumping and the size of the clumps, (Fig. 287) as + + (*strongly positive*), + (*positive*). ± (*doubtful*), and — (*negative*).

EAGLE TEST FOR SYPHILIS [a]

Preparation of Antigen.—Fifty grams of dried powdered beef heart (Difco) are extracted for 15 minutes at 30° to 37° C. with 250 c.c. anesthesia ether, with frequent shaking. The mixture is filtered with suction, the ether extract is discarded, and the powder is similarly extracted with a second portion of fresh ether (250 c c.). This is repeated for a total of *four* extractions. All the ether extracts are discarded. The beef heart powder is then washed on the filter with 100 c.c. fresh ether, thoroughly dried, and extracted with 250 c.c. of *absolute* ethyl alcohol for 3 to 5 days at 20° to 37° C. At the end of this time, the alcohol mixture is filtered, and the moist powder is washed with small portions of fresh absolute alcohol until the combined alcoholic extract and washings measure 250 c.c.

For use in the flocculation reaction, this basic extract is fortified with 0.6% each of cholesterol and corn germ sterol (6 mgs. of each sterol per c.c. antigen). The required amount of sterols are added to a measured volume of antigen, and dissolved by boiling in a water bath at 65° to 85° C. The antigen is then stored at room temperature, and remains serviceable for at least 2 years.

Preparation of Antigen Dilution.—The clear antigen (containing 0.6% each of cholesterol and corn germ sterol) is diluted by rapidly blowing 1.3 volume of 4% NaCl solution into 1 volume of antigen. It is essential that the salt solution and antigen be rapidly and completely mixed. If kept in the ice-box, this dilution remains serviceable for at least 7 days. Because its sensitivity gradually increases during the first 48 hours, the recommended procedure is to prepare the dilution in advance, and allow it to age in the ice-box for at least 24 hours before use.

The Serum Test.—The serum to be tested is inactivated for 20 minutes at 56° C., and 1/15 its volume of the antigen dilution added. Although the test can be carried out with 0.3 c.c. of serum and 0.02 c.c. of antigen dilution, it is more convenient to use twice these quantities (0.6 c.c. serum and 0.04 c.c. antigen dilution). The turbid mixture is then shaken for 2 minutes. The incubation period can be adjusted at will to suit the circumstances.

If a rapid reading is necessary, as for an emergency transfusion, the tube is incubated for one-half hour at 56° C. Otherwise, it is incubated at 37° C. for 4 to 8 hours. After incubation, the tube is centrifuged at approximately 1500 r.p.m. for 10 to 15 minutes. Three volumes of salt solution (0.85% NaCl) are then added, *i.e.*, 3 times the volume of serum used; *and the results of the test are read.*

In a *negative* reaction the tube is seen to be homogeneous and diffusely opalescent. On shaking one sees a swirl of tiny refractile crystals, not visible if the tube is at rest. In a few sera, a little sediment may be deposited on the bottom of the tube, particularly if it was centrifuged at too high speed. This sediment, however, is not coherent and completely redisperses on mild agitation. In a *positive* reaction, the crystals clump to form coherent coarse white flocculcs floating in a clear and transparent fluid. There is a sharp contrast between the water-clear fluid and coarse

floccules of the positive test, and the homogeneous opalescence of the negative test.

One occasionally encounters weak positives, particularly in patients under antisyphilitic treatment. Usually, aggregation in such cases is definite; but even when there is only a slight granular appearance, not sufficiently marked to justify an immediate reading of *positive*, a second centrifugation usually enables one to evaluate the results in terms of positive or negative. If aggregates are really present, they are thrown down in the salt: serum mixture to form a coherent floccule at the bottom of the tube, covered by a clear supernatant fluid, and the result is positive. If no aggregates are present the crystals remain discrete and are not thrown down: the tube remains homogeneous and opalescent. If the results cannot be read as definitely positive or definitely negative even after the second centrifugation, the report is *doubtful*. A slight sediment of discrete crystals which redisperse on slight agitation is ignored.

Although the macroscopic reading is more satisfactory, it is possible to read the results by microscopic examination. In a negative test one sees myriads of tiny crystals which do not cohere even though they are in immediate contact. In a positive test these crystals are clumped in much the same manner that red cells are clumped by an agglutinating serum, leaving clear spaces between the aggregates.

Reports are made as *positive*, *doubtful* and *negative*.

The Spinal Fluid Test.—To 2 c.c. of fresh fluid are added 0.02 c.c. of the antigen suspension. It is to be noted that the fluid-antigen ratio (100:1) is much larger than the corresponding ratio in the case of the serum (15:1). The test is incubated for 4 hours at 37° C., centrifuged for 10 minutes (no salt solution is to be added), and the results read. In a *negative* result, the fluid is diffusely opalescent; in a *positive* result, the fluid is water-clear, and there is a tightly packed lipoid-reagin aggregate at the bottom of the tube. In a *doubtful* result, there is only partial aggregation and sedimentation of the crystalline particles of the antigen suspension.

HINTON TEST FOR SYPHILIS (THIRD MODIFICATION) [7]

This test requires precision in execution because consistent and accurate results cannot be obtained if minor variations in technic are allowed to creep into the routine.

Equipment.—1. Test-tube racks. To simplify numbering and pipetting sera, these racks should be constructed to hold 10 or 20 tubes in a row.

2. Serum tubes 100 mm. long with an approximately uniform inside diameter of 10 mm.

3. A water bath for inactivating sera.

4. A Wassermann bath or a bacteriologic incubator. The former is preferred, because by its use the test is from 3 to 5% more sensitive. The water in the bath should be changed frequently to prevent a deposit from sticking to the outside of the tubes.

5. A centrifuge with a speed of over 2000 revolutions per minute.

[7] W. A. Hinton, *Syphilis and Its Treatment*, Macmillan, New York.

6. A thermometer that registers the maximum and minimum temperatures.

7. Graduated 100 c.c. and 250 c.c. cylinders for measuring the reagents.

8. Dropping pipets with rubber bulbs of about 5 c.c. capacity, for drawing off sera.

9. Serologic pipets of 1.0 c.c. capacity, graduated in tenths to the tip, for measuring the serums, and 5 c.c. or 10 c.c. serologic pipets for measuring the reagents.

10. Thick-walled Erlenmeyer flasks of 125 or 250 c.c. capacity, with an inverted V-shaped ridge blown in the bottom (Fig. 313), for mixing glycerinated indicator. This ridge should produce two semicircular compartments, each of which should hold from 3 to 5 c.c. in flasks with a capacity of 125 or 250 c.c. (Flasks of this type are not listed in any of the catalogues. They may be obtained from Macalaster Bicknell Company of Boston, Massachusetts.)

Cholesterinized Heart Extract.—Extract dried, ground, beef-heart muscle (Bacto-Beef Heart, Dehydrated, Difco Laboratories) by putting 100 gms. of the powder and 400 c.c. of ether (anesthesia) in a wide-mouthed, glass-stoppered bottle and shaking thoroughly by hand for 10 minutes. Allow the bottle to stand 5 to 10 minutes so that the solid material may settle out. Then pour as much of the ether as possible through filter paper into an Erlenmeyer flask, without pouring out any large quantity of the solid material. Scrape the solid material from the filter paper into the bottle for further extractions. Do not allow the main portion of extracted tissue to dry between extractions. Discard the filtrate

Fig. 313.—Special Flask for Diluting Antigen for the Hinton Test

in the Erlenmeyer flask. Make a total of 5 separate extractions using 400 c.c. of fresh ether and a new filter paper for each. After the final extraction, let the residue dry on the filter paper. Obtain the net weight of this dried residue of ether-insoluble constituents. Place in a glass-stoppered bottle with 95% ethyl alcohol in the proportion of 5 c.c. of the alcohol to each gram of residue. Extract for 3 days at room temperature (17° to 20° C.), shaking the contents of the bottle vigorously by hand for 5 minutes 3 times each day. Remove the tissue by filtering into a graduated cylinder; measure the alcoholic extract, and transfer to a glass-stoppered bottle. Add cholesterol in the proportion of 0.4 gm. to each 100 c.c., and warm at 37° C. in an incubator or water bath, occasionally shaking, until the cholesterol has dissolved.

The indicator should not be stored in a refrigerator, for chilling will precipitate

the cholesterol; if, by inadvertent chilling, the cholesterol should precipitate, it must be redissolved in the same manner as that just described. Cholesterinized heart extracts kept in colorless, glass-stoppered bottles at room temperature, for a period of more than 2 years have given as good results as those freshly prepared. Samples of cholesterol obtained from Merck and from the Difco Laboratories were equally satisfactory. All of the many cholesterinized heart extracts prepared in accordance with these directions have given almost identical results.

Five Per Cent Salt Solution.—Prepare a 5% solution of sodium chloride (C.P.) in sterile distilled water, and add 1.0 gm. of salicylic acid (C.P.) to each 4500 c.c.

The salicylic acid helps to preserve the potency of glycerinated indicator (described later).

Fifty Per Cent Solution of Glycerol.—Prepare by mixing equal volumes of Baker and Adamson's Glycerin (Reagent) and sterile distilled water.

The 5% salt solution and the 50% glycerol solution keep indefinitely.

Preparation of Glycerinated Indicator.—This requires strict adherence to the directions for mixing. Thirty cubic centimeters is the smallest and 150 c.c. the largest amount that can be satisfactorily prepared at one time. If larger quantities are required, two or more batches should be pooled. It will remain unimpaired in strength one month and sometimes longer if kept in a refrigerator at a temperature of about 8° C.

The glycerinated indicator is prepared as follows: Pipet one part of the cholesterinized heart extract into one compartment of the Erlenmeyer flask (with the inverted V-shaped ridge), and 0.8 part of the 5% salt solution into the other.

Great care should be used to avoid admixture of the two solutions when the salt solution is pipetted into the flask. A 125 c.c. flask is suitable for the preparation of 30 to 60 c.c. of glycerinated indicator, and a 250 c.c. flask for 90 to 150 c.c.

Mix by shaking the flask very rapidly from side to side for 1 minute. Let the mixture stand exactly 5 minutes. Without further delay add 13.2 parts of the 5% salt solution and shake thoroughly. Finally, add 15 parts of the 50% glycerol solution and shake until the suspension is homogeneous.

Procedure.—1. Centrifuge the blood, if necessary, to aid in separating the serum from its clot, and with a long, dropping pipet remove the serum (free from blood cells) and deliver into an appropriately labeled serum tube.

To avoid contamination of one serum by another, after each has been drawn off, the dropping pipet should be thoroughly rinsed at least 3 times with sterilized physiologic salt solution; and to minimize bacterial contamination, after every 20 sera have been drawn off, the washing bottle (about 200 c.c. capacity) should be emptied and filled with fresh salt solution.

2. Heat the sera in the inactivating bath at 55° C. for 30 minutes. Be sure that the level of the water in the bath is above the level of every serum and that the temperature is kept at 55° C. or 56° C. throughout the period of inactivation. Errors may result if this varies even one or two degrees. Sera should be inactivated

the day of testing. If it is necessary to retest a specimen, use serum freshly separated from the clot.

3. Select all serums that show (a) hemolysis, as manifested by redness greater than that produced by dissolving 0.1 c.c. of blood in 3.0 c.c. of distilled water; (b) bacterial contamination, as shown by cloudiness; or (c) marked opacity from other causes. Place in a separate rack and test, as soon as conveniently possible, according to the quick method (page 000). This is done to avoid further deterioration, which decreases the sensitiveness of the test and makes it harder to read.

4. Set up the racks with one properly numbered serum tube for each of the remaining specimens. Tubes should be clean and clean. To clean the tubes, rinse them thoroughly, as soon after use as possible, with tap water, and then fill each with a warm solution of 5 gms. of sodium hydroxide in 1000 c.c. of tap water; allow them to stay in this solution for about 2 hours and wash thoroughly with hot water to remove the alkali. This process usually removes (without the aid of a test-tube brush) any deposit which may have remained from previous use.

5. With a 1.0 c.c. pipet, measure 0.5 c.c. of each heated serum into the tube that has been labeled for it. Use a separate pipet for each serum.

For routine purposes one tube for each test is sufficient. If, however, this test is negative and there is reason to suspect syphilis it is desirable, to retest the specimen using 0.1 c.c. in one tube and 0.5 c.c. in the other. In approximately one syphilitic out of 200, the tube containing the 0.1 c.c. of serum gives a positive reaction, while the one which contains the 0.5 c.c. of serum gives a negative reaction.

Positive and negative controls should be used if only a few tests are to be made at one time.

6. Compare the appearance of each of the pipetted serums with that in the tube from which it was taken. This is to make sure that there has been no error in pipetting or in labeling the tubes.

7. Not more than 30 minutes before incubation, with a clean 10 c.c. pipet, add 0.5 c.c. of the glycerinated indicator to each serum.

8. Pipet 0.5 c.c. of the same indicator and 0.5 c.c. of the 5% salt solution into an empty serum tube. This serves as a control, the use of which will be indicated later.

9. Incline the rack to an angle of about 45 degrees and then shake by thrusting it quickly upward and forward, then downward and backward with sufficient speed to cause the fluid to travel halfway up the tubes and make small bubbles. At least 3 minutes of shaking are required for accurate results. If there are enough tests a shaking machine is desirable.

10. Place the rack in the Wassermann bath or incubator at 37° C. and let it remain for 16 hours (conveniently from 5 P.M. to 9 A.M.), or in the incubator for 18 hours. Longer incubation makes the tests increasingly hard to read. Do not agitate the contents of the tubes before reading.

When the racks containing the tests are removed from the bath or incubator, record the readings shown by the bath or incubator thermometer as well as those

of the maximum and minimum thermometer. For dependable results the temperature should not fall below 34° C. nor rise above 39° C.

Reading the Tests.—The tests are easier to read within an hour after the incubation than later. To read them, sit in front of a window, but do not face the sunlight. The light must be good, and for this reason suitable artificial light must be provided on dark days or at night. In order to determine whether or not there is clearing of the fluid and a ring or band of white flakes or white coarse granules at the meniscus, lift each tube carefully from the rack, hold it at the level of the eye, slant it at an angle of about 45 degrees, and view it in the direction of a darkened background on either side of a window or of a suitably placed light. While still viewing it at the same angle, slowly rotate the tube by rolling it between the fingers; this will make even a faint ring visible. Finally, gently shake the tube and look for a precipitate which may manfest itself by agglutinated masses or by only very faint granularity.

The reactions are read and reported as positive (without indicating the intensity of the reaction), negative, doubtful and unsatisfactory.

The simplicity of this method of reporting has the advantage of not confusing physicians by implying that the test is in any way a quantitative reaction, for the intensity of the reaction actually bears no relation to the clinical condition of the patient.

Positive Tests (*Recorded* +).—At or a little above the level of the meniscus there is a ring or band, approximately 0.2 to 1.5 mm. wide, of white coarse granules or flakes of lipoids slightly to moderately, but not strongly adherent to the wall of the tube. The gentle shaking loosens the ring or band and scatters the particles so that they are visible as agglutinated masses in a clear fluid or as somewhat coarse granules in a cloudy fluid.

Negative Tests (*Recorded* —).—There is at most only slight clearing, but no ring, band or precipitate.

Doubtful Tests (*Recorded* ±).—Centrifuge for 10 minutes at high speed (about 2000 revolutions a minute) (1) those tubes which on gentle shaking showed only slight granularity beyond that observed in the control tube (containing 0.5 c.c. of indicator and 0.5 c.c. of the 5% salt solution, and (2) those tubes which showed only a slightly flakey or slightly granular ring. If, as a result of the centrifuging, there is definite clearing, and on top a thin layer of lipoids which on shaking breaks up into fine flakes or coarse granules, the test is reported as doubtful and recorded ±; if these changes have not taken place, it is reported as negative.

Quick Method.—Treat the tests selected under No. 3 as indicated under directions 4, 5 and 6. Then add 0.5 c.c. of the glycerinated indicator to each, and shake according to direction 9; next, centrifuge at 1500 to 2000 revolutions a minute for ten minutes, read, and record as follows:

The test is positive (recorded +) if there are plainly visible flakes at the top of the fluid, and a well-marked precipitate is seen on shaking. Hemolyzed and bacterially contaminated specimens are recorded and reported as unsatisfactory unless the reaction is strongly positive. This interpretation is necessary because even the

moderately hemolyzed or bacterially contaminated serum of a known syphilic usually gives a negative reaction. In the absence of hemolysis or bacterial contamination the test is negative (recorded —) if the centrifuging has caused no change.

Whenever there is immediate need for a report, this quick method may be used for it will detect about 80% of the positive reactions obtainable by the regular test. Tubes that show only a very fine precipitate or no precipitate at all after the centrifuging should be well shaken and placed in the water bath for 16 hours, or in the incubator for 18 hours, after which the reading and interpretation are made as if the tests had been conducted in the routine manner.

THE PRECIPITATION TEST FOR THE IDENTIFICATION AND DIFFERENTIATION OF BLOOD STAINS AND OTHER PROTEINS

These tests are usually required for medicolegal purposes and especially for the identification of human blood.

Preparation of Immune Serum.—1. Immunize several rabbits by *intravenous* injections of human serum at 3-day intervals in the following amounts: 1, 2, 3, 4, 8, and 10 c.c. or inject at 5-day intervals in the following amounts: 2 doses of 8 c.c. each, two of 5 c.c., and two of 3 c.c. To prevent anaphylactic shock the rabbits may be desensitized by injecting 0.2 c.c. of the serum one-half hour before injection of each of the later doses. Ten days after the last dose remove a small amount of blood from an ear vein and test the serum for precipitin by placing 0.2 c.c. in a small test tube and carefully overlaying with 1 c.c. of a 1 : 1000 dilution of human serum. If a ring of precipitation occurs in a few minutes the *fasting* animal may be bled from the heart aseptically in sterile centrifuge tubes. After clotting has occurred, *gently* separate the clot and centrifuge to remove all corpuscles. Transfer the serum to sterile containers and to each 19 c.c. add 1 c.c. of 1 : 500 metaphen solution for preservation. For use the serum must be crystal clear and this may require centrifugalization or filtration.

If the preliminary test is negative give the animal three additional doses of serum *intraperitoneally* in the following amounts: 10, 15 and 20 c.c. Test again 10 days after the last dose.

For medicolegal work it is advisable to prepare similar precipitins for the sera of the commoner domestic animals as the hog, sheep, beef, chicken, dog, cat, etc.

Preparation of Unknown Material.—Prepare a solution of the stain or other material in normal saline solution. Apply a test for blood like the benzidine, hemin crystal or other procedure. The extract should be approximately 1 : 1000 and fulfill the following requirements: (a) crystal clear and almost colorless; (b) contain only a very small amount of protein when tested by the heat and acetic acid or nitric acid ring tests; (c) foam freely on shaking; (d) be neither strongly acid nor strongly alkaline to litmus paper.

If the solution of stain was from a piece of cloth, leather, or other material prepare a similar extract of an unstained portion as a control. Of course, it may not contain protein.

Method.—1. Arrange 7 small test tubes or preferably pointed capillary tubes of about 3 mm. inside diameter and charge them as follows:

No. 1.—0.2 c.c. immune serum overlayed with 1 c.c. of unknown extract.

No. 2.—0.2 c.c. normal rabbit serum overlayed with 1 c.c. of unknown extract (negative control).

No. 3.—0.2 c.c. of immune serum overlayed with 1 c.c. of a 1:1000 solution of sheep or other serum different from that suspected in the stain of unknown material (negative control).

No. 4.—0.2 c.c. immune serum overlayed with 1 c.c. of 1:1000 dilution of human serum (positive control).

No. 5.—0.2 c.c. immune serum overlayed with 1 c.c. of control extract if one has been prepared of unstained cloth, leather, etc. (control).

No. 6.—0.2 c.c. immune serum plus 1 c.c. of normal saline solution (control).

No. 7.—0.2 c.c. saline solution plus 1 c.c. of extract (control).

2. Allow to stand at room temperature for 5 to 15 minutes and examine.

3. White rings of precipitation at the lines of contact in tubes 1 and 4 with no reactions in the other tubes indicates that the extract of unknown material contained human protein and if the chemical reactions were positive for blood would indicate that the extract was of human blood. The test, however, does not differentiate the blood of one human being from another. Doubt can arise only between the proteins of closely related species, as, for example, man and the higher apes, sheep and goat, horse and mule, etc.

4. If the tests are negative for human blood duplicate tests may be conducted with anti-chicken, anti-beef, anti-dog, anti-cat and other immune sera. In medicolegal cases it is always advisable to conduct these even though positive reactions are observed with anti-human serum.

5. Similar tests can be conducted for the detection of meat adulteration, identification of bones, milk, semen, etc., the technic being given in Kolmer's *Infection, Immunity and Biologic Therapy,* W. B. Saunders Company.

PRECIPITIN TEST FOR PLAGUE

1. This test is sometimes of value in making a rapid presumptive diagnosis on decayed tissues and especially of rats.

2. One part of finely divided tissue is mixed with 5 to 10 parts of distilled water, boiled for 5 minutes and filtered repeatedly through paper or asbestos wool with suction until perfectly clear. Set up the tests in small test tubes:

No. 1.—Place 0.5 c.c. of high titer immune serum and carefully overlay with 0.5 c.c. of the filtrate.

No. 2.—Set up a duplicate using normal rabbit or horse serum (control).

No. 3.—Place 0.5 c.c. of immune serum and overlay with 0.5 c.c. of normal saline solution (control).

No. 4.—Place 0.5 c.c. of extract and overlay 0.5 c.c. of normal saline solution (control).

3. A positive reaction shows a white ring of precipitate at the line of contact in the first tube in 5 minutes at 37° C. reaching a maximum after 2 hours. A negative reaction is not conclusive.

PRECIPITIN TEST FOR ANTHRAX

A similar test may be conducted with an extract of tissue and a potent anti-anthrax serum.

PRECIPITIN TEST FOR ECHINOCOCCUS DISEASE

This test is frequently of diagnostic aid. The antigen is the clear fluid from a hydatid cyst. The serum is that of the patient which should be crystal clear, undiluted and used unheated.

1. Set up the tests in small test tubes as follows:

No. 1.—0.5 c.c. of patient's serum carefully overlayed with 0.5 c.c. of antigen.

No. 2.—0.5 c.c. of normal serum carefully overlayed with 0.5 c.c. of antigen (negative control).

No. 3.—0.5 c.c. of serum from a rabbit immunized by intravenous injections of cyst fluid or from a known case of the disease carefully overlayed with 0.5 c.c. of antigen (positive control).

No. 4.—0.5 c.c. of patient's serum with 0.5 c.c. of saline solution (control).

No. 5.—0.5 c.c. of antigen with 0.5 c.c. of saline solution (control).

2. Allow to stand at room temperature for one-half hour. A white ring of precipitation in tubes 1 and 3 indicates a positive reaction.

PRECIPITIN TEST FOR MENINGOCOCCUS MENINGITIS
(KREIDLER AND MURPHY)

This test is not only of diagnostic aid in differentiating meningococcus from other types of suppurative meningitis, but it is also of service in the selection of antimeningococcus serum for treatment, preference being given to the serum showing the maximum of precipitation.

1. Centrifuge the spinal fluid at high speed for 20 minutes.

2. Remove the clear supernatant fluid to another test tube. The sediment may be used for smears and cultures.

3. Place 0.3 to 0.5 c.c. of each available antiserum in small test tubes.

4. Carefully overlay each with an equal volume of the clear spinal fluid.

5. Allow to stand at room temperature for 20 minutes. Examine for rings of precipitation at the lines of contact which constitute positive reactions graded from + to + + + +.

CHAPTER XXXI

METHODS FOR CONDUCTING TESTS FOR ALLERGY

While the diagnosis and treatment of diseases due to allergy is properly a specialty in clinical medicine, yet certain laboratory examinations are sometimes required such as total and differential leukocyte counts for eosinophilia: the detection of eosinophilia in smears of nasal, sinus, bronchial, conjunctival and intestinal secretions; the preparation of allergenic extracts and vaccines for skin testing and finally the conduct of skin tests, sometimes requested of the clinical pathologist and laboratory technician.

METHODS FOR THE PREPARATION OF ALLERGENS

Prepared extracts of a very large number of different substances as pollens, hairs, dandruffs and feathers of the lower animals, various foods, silk, cotton, glue, orris root, etc., may be obtained from manufacturing laboratories suitable for the scratch or cutaneous test. These are usually in the form of powders or solutions.

Fluid extracts of allergenic substances may be obtained in the same manner suitable for intracutaneous tests as well as the allergenic oils of pollens, poison ivy, sumac, oak, etc., for the patch test. It is unnecessary, therefore, to include here the technic of their preparation.

Stock dust extracts may be likewise obtained from the same sources, but autogenous extracts prepared of house or industrial dusts to which the individual patient is exposed, are sometimes required. These, as well as extracts of foods or other materials, may be prepared as follows for intracutaneous tests and treatment by desensitization:

Principles.—1. The extract should contain sufficient of the active excitant or allergen to give positive skin reactions in sensitive individuals.

2. It should be prepared with the minimum of denaturing of the active allergen. This is probably of special importance with foods. In the case of foods eaten in the raw state it is preferable to prepare extracts of the raw material. In the case of cooked foods it is preferable to prepare the extracts after the usual amount and degree of cooking as it would appear possible for an allergic individual to give a negative reaction to an extract of raw food and a positive to an extract of the same food prepared after cooking; likewise, the reverse.

3. It should be sterile and nonirritating to the skin.

4. It should be of such strength that the test dose does not produce nonspecific reactions in normal nonallergic individuals.

Method.—1. Divide the food very finely and include the juices. The wet food may be extracted or it may be dried and pulverized. With dust (collected in a

vacuum cleaner from the floors, rugs, draperies and mattress), remove match sticks, hairpins, etc.

2. Place the material in a flask and add 2 or 3 volumes of carbon tetrachloride. Stopper tightly and shake thoroughly. Place in incubator at 37° C. for 1 or 2 days. Decant and discard the carbon tetrachloride and add 2 or 3 volumes of water-free ether. Stopper tightly, shake and keep at room temperature for 1 or 2 days with frequent shakings. These measures are for the removal of fats and coloring matter.

3. Filter through paper and allow the material to dry on the paper overnight.

4. Transfer the material to a flask and add an equal volume of one of the following extracting fluids:

COCA'S EXTRACTING FLUID

Sodium chloride	4.0 gm.
Sodium carbonate (Na₂CO₃)	1.4 gm.
Phenol (5%)	40.0 c.c.
Distilled water to make	500.0 c.c.

When 2 or 3 drops of a 1% alcoholic solution of phenolphthalein are added to 5 c.c. it should remain colorless; to insure this state Coca advises passing carbon dioxide through the fluid before use.

BUFFERED SALINE SOLUTION

Sodium chloride	50.0 gm.
Potass. dihydrogen phosphate	3.63 gm.
Disodium hydrogen phosphate	14.31 gm.
Distilled water	1000.0 c.c.

Mix equal parts of this solution with 4% solution of phenol. For use dilute 1 part with 4 parts of sterile distilled water.

5. The Coca fluid is advised for the preparation of extracts of pollens and dusts; the buffered saline for all other extracts. The latter is also used for preparing dilutions of extracts.

6. Cover with a thin layer of toluol to inhibit bacterial activity and extract in the incubator for 48 hours.

7. Filter through paper, gently squeezing the material. The filtrate may be concentrated by evaporation in a large dish exposed to an electric fan while carbon dioxide is bubbled through or by ultrafiltration through cellophane.

8. Pass the filtrate through a sterile Berkefeld, Mandler or Seitz filter. It is advisable but not necessary to standardize by determining the total nitrogen by the Kjeldahl method or the protein nitrogen by the phosphotungstic acid precipitation method. As a general rule satisfactory standardization may be accomplished by testing normal individuals with intracutaneous injections of 1:5, 1:10, 1:100, etc., to determine the lowest dilution giving a completely negative reaction.

9. Culture for sterility by placing 2 or 3 c.c. in a flask of 100 to 200 c.c. of broth (pH 7.2) and incubate at least 5 days.

10. Keep the extract in a refrigerator where it usually maintains allergenic activity for about one year. If in bulk, cover with a thin layer of toluol.

Notes.—1. Hairs and dandruffs are apt to be particularly greasy and require thorough de-fatting. Extract with buffered saline solution.

2. Feathers should not have been previously washed or immersed in water.

3. With cereals cover the extracting fluid with toluol. Use minimum amount of extracting fluid. Dialysis of the extract aids in removing viscosity and makes filtration easier (also applies to orris root).

4. Nuts, seeds and beans should not be roasted; grind in a coffee grinder and thoroughly remove the oils with ether before extracting. Dialysis aids in removing irritating substances.

Method for Milk (Coca).—1. Remove all fat from 500 c.c. by centrifuging.

2. Add 2.5 c.c. of 1% rennin and place in a water bath at 37° C. for one-half hour without stirring.

3. Remove the precipitated casein by straining through a sterile towel.

4. Add 7 c.c. of a saturated solution of sodium carbonate to the filtrate.

5. Filter through a sterile Berkefeld, Mandler or Seitz filter and culture for sterility.

Method for Eggs.—Dilute whole egg white 1:10 and 1:100 with the buffered saline solution. Sterilize under toluol for three or more days. Culture for sterility.

TECHNIC OF SKIN TESTS

Choice of Methods.—Two methods are employed, namely; (a) the *cutaneous* or scratch test and (b) the *intracutaneous* test. Both have certain advantages and disadvantages so that it is not so much a choice between the two but rather the use of either or both according to conditions.

The cutaneous test appears satisfactory for the detection of allergy to inhalants, as in hay fever and asthma. It is cheap, safe, quickly conducted and highly specific. Twenty to 40 tests may be done at one time.

The intracutaneous test, however, is more sensitive and especially required for the detection of allergies to the foods and bacteria. It is more likely to give non-specific reactions in inexperienced hands. The reactions are more definite and easier to read and while easy to perform, only a limited number as 6 to 12 can be done at one time.

A good general rule is to conduct cutaneous tests first and to repeat with intracutaneous tests in the case of those substances giving negative cutaneous reactions.

Diagnostic Value of Skin Tests.—1. Skin tests are seldom required for the diagnosis of allergy as this is usually possible by a thorough history and physical examination.

2. Their chief value is the aid they render in detecting the exciting agent or agents of allergy.

3. Both cutaneous and intracutaneous tests may give falsely negative reactions.

These may be due to technical errors or to the fact that the skin is not sensitized.

4. Both cutaneous and intracutaneous tests may give positive reactions to substances to which the patient is not clinically sensitive. This is sometimes called "potential allergy."

5. In general terms a positive skin reaction to a certain substance simply means that the skin is specifically sensitive to it. But this does not necessarily imply that it is of clinical or etiological importance. In the great majority of instances, however, positive reactions have much more diagnostic import than negative reactions.

6. In conducting skin tests there should be a very careful selection of allergens in order to omit unnecessary tests. In the food tests for example it is usually well to avoid those the patient rarely or never ingests.

7. Group tests are not advisable. They are usually combinations of 4 or more foods or other substances and have been advocated to reduce the number of tests. However, they may give falsely negative reactions. When positive reactions are observed it is necessary to repeat the tests with the individual foods.

TECHNIC OF THE CUTANEOUS OR SCRATCH TEST

1. Make a careful selection of the allergens to be tested.

2. Cleanse the forearm with alcohol and allow to dry. The arm, back or thighs may be used but only in case of necessity.

3. It is necessary to make a superficial cut or scratch for each allergen to be tested. Ordinarily there can be two rows of 10 to 12 each along with one control. These scratches should be about one inch apart and about ¼ inch in length. They should be deep enough to draw serum but not blood. They may be made with any sharp instrument, as a Daland blood lancet (Fig. 314) or scalpel cleaned with alcohol.

4. It is a good plan to prepare a diagram of the forearm marking down the name of each allergen opposite its scratch in order to avoid errors or confusion.

5. If the allergens are in powder form, proceed as follows:

(a) With a toothpick place a drop of decinormal NaOH solution or normal saline solution on a scratch; (b) add a small amount of allergen and rub in gently for several seconds (Fig. 315); (c) with a fresh toothpick repeat with the next allergen, etc.; (d) the control scratch is rubbed in the same manner with the solution used for dissolving the allergen.

6. If the allergens are in fluid or paste form it is only necessary to add a drop to a scratch with gentle rubbing.

7. Wait 20 to 30 minutes before gently washing off the stains and making the readings.

8. First examine the control. Some individuals and especially children or adults with urticaria may show excessive reactions to the trauma of the scratch and fluid. To be positive a reaction must be definitely larger than the control, that is, show a larger wheal with a larger areola. The typical positive reaction looks like a
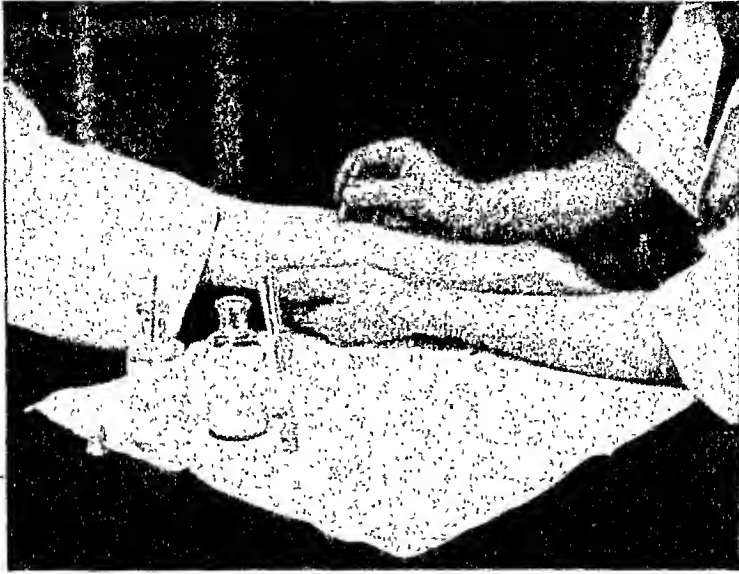
FIG. 344.—METHOD OF MAKING SACRIFICATIONS FOR CUTANEOUS ALLERGIC TESTS WITH DALAND LANCET

(From Kolmer, *Infection, Immunity and Biological Therapy*, 3 ed., W. B. Saunders Co.)
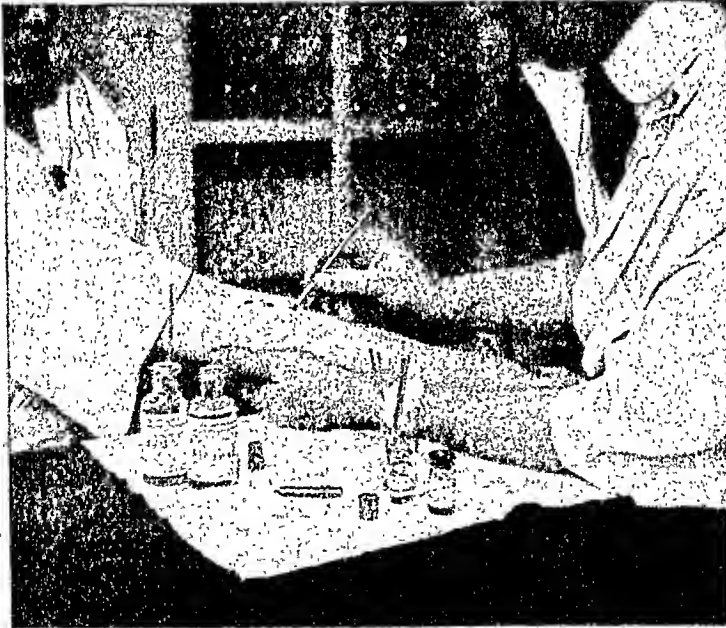
FIG. 345.—METHOD OF APPLYING ALLERGENS TO CUTANEOUS ABRASIONS IN ALLERGIC SKIN TESTS

(From Kolmer, *Infection, Immunity and Biological Therapy*, 3 ed., W. B. Saunders Co.)

"live" or mosquito bite with pseudopodia and pinkish areola (Plate XII) and according to size, etc., may be recorded as follows:

— Negative
± Wheal less than 0.5 cm.; moderate areola
+ Wheal 0.5 cm.; moderate areola
+ + Wheal 0.5 cm.; marked areola
+ + + Wheal 0.5 to 1 cm.; with or without marked areola
+ + + + Wheal above 1 cm.; with or without marked areola

Reactions are sometimes delayed up to 24 hours but their exact significance is not known.

TECHNIC OF THE INTRACUTANEOUS TEST

1. Make careful selection of extracts to be tested. If the patient is suspected as being highly sensitive, the number of tests should not ordinarily exceed 6 to 12. Otherwise, a large number may be done if the discomfort is not too great.

2. The injections must be strictly intracutaneous and are best made with ¼ inch gage No. 26 or 27 needles fitted to tuberculin or other type of small syringes. It is a good rule to have a separate syringe for each allergen. Syringes and needles must be carefully sterilized.

3. The arm is preferred although the forearm or back may be used if necessary. Cleanse with alcohol and cotton.

4. The injections should be at least one inch apart and, in adults, can be arranged in two rows of 4 to 6 injections.

5. A control injection of diluting fluid alone is required.

6. The skin may be pinched in a fold or stretched and the needle entered with bevel upward. The dose injected is usually 0.01 or 0.02 c.c. Slight stinging is felt and an anemic spot produced (Fig. 316). If the latter is not seen the injection has been too deep.

7. Reactions are usually read in about 10 minutes and may be classified as follows: (a) negative (resembles the control); (b) slightly positive (+) due to slight increase in either the size of the wheal or area of erythema or both; (c) moderately positive (+ +) with moderate increase in the size of area of erythema and wheal but no pseudopodia; (d) markedly positive (+ + +) with large wheal and areola, pseudopodia and itching.

8. In some instances reactions are delayed for several and even 24 hours with a tendency to persist longer than immediate reactions. Their exact significance is unknown but in the food allergies are sometimes of significance.

TECHNIC OF THE PATCH OR CONTACT TEST

This test is employed in the determination of the specific exciting agent responsible for certain inflammatory conditions of the skin grouped under the term of contact dermatitis and best illustrated by poison ivy. It is the only test of any value in the diagnosis of these conditions and is at times an invaluable aid.

PLATE XII.

Control

Ragweed

Golden rod

Orchard grass
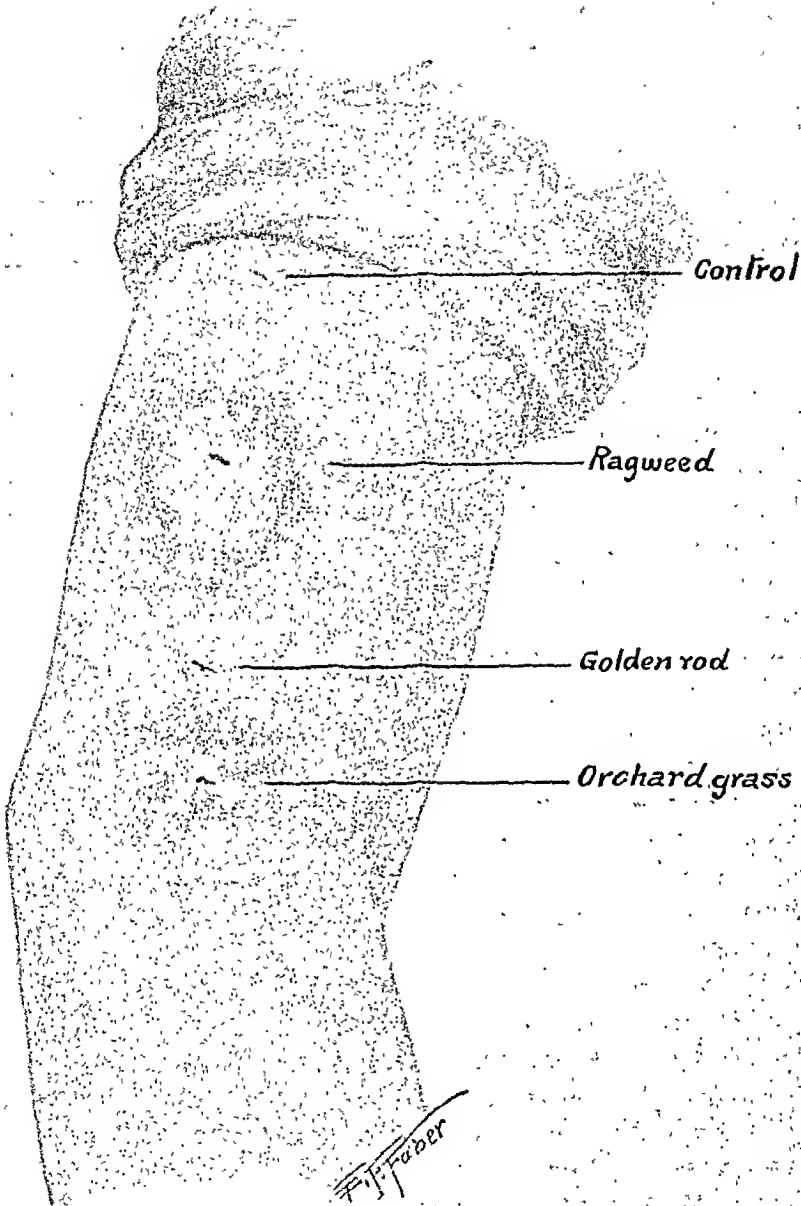
POSITIVE ALLERGIC REACTIONS TO POLLENS
(cutaneous or scratch tests)

(From Kolmer, *Infection, Immunity. and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

Principle.—Since the eruption of contact dermatitis is the result of an acquired sensitization, the direct application of the suspected excitant to the skin of such a patient should, after sufficient contact, reproduce a lesion similar to that for which he patient is being treated.

Technic.—1. Prepare a patch consisting of adhesive plaster 1—2 inches square. Cover all of the inner or adhesive surface of the patch except for a rim of about ¼ inch with a square of cellophane, rubber tissue or oiled silk.

2. Prepare a small square (½ inch) of linen or blotting paper to be used for

Fig. 346.—Technic of Intracutaneous Injection

The skin has been cleansed with alcohol and pinched up between the thumb and index-finger of the left hand; the needle (No. 26) has been entered into the epidermis and 0.1 c.c. of fluid injected. Note the anemic area, indicating that the injection has been intradermic. (From Kolmer, *Infection, Immunity and Biologic Therapy*, 3 ed., W. B. Saunders Co.)

absorbing the liquid or ointment preparations. Solid substances may be applied directly to the skin.

3. The skin of the outer surface of the arm or inner surface of the forearm should be employed when only a few tests are to be done at one time. When 10 to 20 are applied. the skin of the back is more convenient.

4. To perform the test, soak the small square of linen or blotting paper in the liquid to be tested and apply it directly to the skin. If the suspected agent is an ointment. this may be put on the square intact; if it is a powder, it may be moistened with saline or distilled water and then put on. Solid materials may be put on

directly without any square. Cover the square with the patch, prepared as described. If the edges of the patch are not firm enough, seal them by adhesive strips or collodion.

5. Allow the patch to remain in place 24 hours, unless itching is marked. With less potent excitants it may be allowed to remain 48 hours or longer.

6. A positive reaction is indicated by the presence at the site of the patch of a reddened area in which small blisters or vesicles are seen. A negative reaction shows no changes at the site. The area should be inspected daily for two weeks for signs of a reaction, before being considered negative.

7. A positive reaction is definite indication of sensitization. Its clinical importance must, however, be established. A negative reaction does not necessarily exclude the presence of sensitization.

TESTS FOR SERUM ALLERGY

These are frequently required before the administration of immune sera for the prophylaxis and treatment of disease, to determine if the patient is hypersensitive.

1. If the patient is an asthmatic and especially if suspected of being sensitive to horse serum, a scratch or *cutaneous* test should be first conducted with a drop of normal horse serum. A positive reaction indicates such a high degree of sensitiveness that serum should not be given at all or only by very special methods.

2. If the history is negative for asthma, or if this test gives a negative reaction, inject *intracutaneously* 0.02 c.c. of sterile normal horse serum (0.2 c.c. of 1 : 10 dilution). A control injection of sterile saline solution is advisable. Read reactions in about 10 minutes. A positive reaction indicates that if serum is given precautions are required and especially if administered intravenously. The test is sometimes conducted with the immune serum for administration. This is not advisable because the antibodies in the serum may produce an immediate erythematous and edematous reaction (known as the *E-E reaction*) which may be mistaken for a positive allergic reaction (Foshey, L.: *Jour. Allergy*, 1935, 6, 360).

3. An *opthalmic* test may be conducted by placing a drop of 1 : 10 dilution normal horse serum in the lower conjunctival sac. The test cannot be conducted in the case of crying children. Wait about 10 minutes. A positive reaction is shown by erythema, lacrimation and some itching. It is not as reliable as the intracutaneous test.

TESTS FOR BACTERIAL ALLERGY IN RELATION TO VACCINE THERAPY

1. Cultures are made on Petri plates of hormone blood agar and the different bacteria isolated and identified.

2. Pure cultures of these are grown on appropriate solid media for 24 to 48 hours and removed with sufficient saline solution to give dense suspensions of at least 2,000,000,000 per c.c. Or the bacteria may be grown in a fluid medium and secured by centrifugation. If any medium has been carried into the suspension it is filtered through sterile paper and the bacteria are washed once with saline solution containing 0.5% phenol before being resuspended in the saline solution.

3. Each suspension is heated in a water bath at 60° C. for one hour, cultured for sterility, and preserved with sufficient phenol to make 0.5%.

4. For the skin tests a small amount of each suspension is diluted with sterile saline solution to give approximately 500,000,000 per e.c.; 0.05 c.c. of each is injected intracutaneously. A control injection of saline solution is made at the same time.

5. The reactions are read approximately 1 and 24 hours later.

6. Those bacteria causing positive reactions are then incorporated into an autogenous vaccine prepared from the stock suspensions.

THE FREI TEST FOR LYMPHOGRANULOMA INGUINALE

1. The *antigen* is prepared by aspirating pus' with a gage No. 18 needle from a softened but unopened bubo. The subject should be known to be free of tuberculosis, syphilis and chancroid. The blood-free pus is then diluted with 5 or 6 parts of sterile saline solution and heated at 60° C. for 2 hours on the first day and for 1 hour on the second. When proven sterile by aerobic and anaerobic cultures the antigen may be put up in ampules ready for use.

Antigen may be prepared by inoculating mice intracerebrally with pus. Immediately after death the brain is removed aseptically and prepared in a paste to which is added 4 e.c. of Savita broth with constant grinding. The emulsion is then sterilized in the same manner as above, cultured for sterility and put up in ampules ready for use. A control antigen of normal mouse brain is advisable.

2. The *test* is conducted by injecting 0.1 e.c. of the antigen intracutaneously in the forearm. A control injection may be given in the other arm.

3. A *positive* reaction occurs as an inflammatory papule appearing in 24 hours and reaching its height in about 72 hours.

INDIRECT METHOD OF TESTING FOR ALLERGY

Principles.—1. This test depends upon the possibility of sensitizing a local area of skin of a normal individual by the intracutaneous injection of the serum of an allergic individual carrying skin-sensitizing antibodies or allergens as in hay fever or asthma (Prausnitz-Kustner method of passive transfer of antibody). This test cannot be done if the allergic individual is syphilitic. Remove 5 c.c. of blood from the allergic individual with aseptic precautions. Separate the serum and centrifuge if necessary to remove corpuscles. Keep in refrigerator. It may be preserved by the addition of 0.1 c.c. of 5% phenol per cubic centimeter. It is advisable to culture for sterility.

2. With a sterile tuberculin syringe fitted with a No. 26 needle, inject 0.1 e.c. of the serum *intracutaneously* into the forearm of a normal nonallergic individual. As a general rule 8 injections may be made in two rows of four each. Do not use senile skins; avoid sunburned areas. If foods are being tested the individual should abstain from these for 48 hours and especially in the case of eggs, fish and nuts. Ring each site of injected skin with ink or skin pencil.

3. Allow 48 and preferably 72 hours to elapse and reinject the *sensitized sites*

with 0.02 c.c. of sterile solutions of the allergens being tested; at the same time inject a similar amount of each into adjacent areas of nonsensitized skin as controls. Also inject 0.02 c.c. of normal saline solution into a sensitized area as an additional control.

4. Inspect both areas 10 minutes later. A reaction in a sensitized site with no reaction in the control site is positive. If both are negative it is likely that the serum had no antibodies for the allergen employed. If both are positive the individual has an allergy to the allergen and the test would have to be repeated with another individual.

METHOD FOR THE DETECTION OF EOSINOPHILIA IN THE DIAGNOSIS OF ALLERGY

1. This method is conducted with smears of secretions from the nose, nasal accessory sinuses, conjunctivae, intestinal tract and bronchi.

2. These may be prepared on slides in the same manner as blood films.

3. Dry in the air; fix with methyl alcohol for 5 minutes and stain with Wright's stain in exactly the same manner as blood.

4. Wash in water, dry and examine with oil-immersion lens.

5. Make a differential count of the cells and calculate the percentage of eosinphils. An excess (25% or higher) is presumptive evidence of allergy.

SECTION V

CHEMICAL METHODS

CHAPTER XXXII

METHODS OF COLORIMETRY, NEPHELOMETRY AND SCOPOMETRY

Principles.—1. *Colorimetry* depends upon comparing and measuring the color of a fluid under examination with a similar solution of known strength, upon the principle that the depth of color is directly proportional to the amount of the substance present.

2. *Nephelometry* depends upon measuring the density of precipitates and thus determining the amount of any substance which can be obtained in the form of a suitable suspension. It differs from colorimetry in that it uses reflected instead of transmitted light. The brightness of two fields is compared instead of their colors. It is adapted particularly for the determination of substances that are in very dilute and colloidal suspension, which do not precipitate appreciably in the time required for making readings. The method has been adapted to the determination of a large number of substances and is continually finding new applications. It is possible to determine very minute amounts of substances, entirely outside of the range of gravimetric methods of analysis by nephelometry, which thus may be used where the amount of material is very small. If properly carried out the limits of error are not greater and may be less than those of the colorimetric methods commonly employed.

3. *Scopometry* is closely related, but depends upon an extinction or vanishing point criterion as an optical measure of turbidity and color, measuring the optical density of a sample when a point is reached at which the density of the sample plus the density added to it cause the image of a standard target to disappear. For adding density to the sample the Exton Junior Scopometer utilizes a continuously graded series of increasing densities in the form of an optical wedge having a predetermined standard slope and called neutral because it transmits equally all colors of the spectrum. By shifting the position of the wedge, the point on the wedge scale at which the target image seen through sample and wedge vanishes directly measures the concentration of the specimen. The results are usually easily read, of considerable accuracy and the methods particularly applicable to small amounts of test material.

COLORIMETRIC METHODS

Duboscq Method.—1. The general construction is shown in Figure 347.

2. The solutions to be compared are placed in glass cups, which can be raised

by means of rack and pinion until the lower ends of the clear glass plungers are immersed in the fluid, the excess of fluid rising between the plungers and the walls of the cups. By raising or lowering the cups the layer of fluid between the lower ends of the plungers and the bottom of the cups may be made of any desired thickness and the thickness of each is indicated by a scale placed in a convenient position. Beneath the cups is a mirror which reflects light up through the cups and the long axis of the plungers into a series of prisms. These reflect the light from the two cups into a single field which is viewed by an eye lens. Each lateral half of the field receives its light through one of the cups. The raising or lowering of the cups, by diminishing or increasing the thickness of the layer of fluid through which the light passes, diminishes or increases the depth of color of the corresponding half of the field.

3. Adjustment of the scales should be tested before an instrument is used. Raise the cups gently until their bottoms are in contact with the plungers. If the reading is not exactly zero on each side, the scales, which are movable upon most instruments, must be brought to accurate adjustment. In the absence of an adjustable scale, a correction must be made in the readings

Fig. 347.—Small Duboscq Colorimeter

on each side, sufficient to indicate the true depth of liquid between the bottoms of the plungers and the top surfaces of the cup bottoms.

4. Test the equal transmission of light through both sides of the instrument by placing the same colored solution, usually a standard, in both cups, taking care that they are not filled so much that displacement of fluid by the plungers causes some to be spilled. Raise the cups until both plungers are immersed, and are set to have the same depth. So place the colorimeter with respect to the light source that the same amount of light is reflected up through each cup. Either daylight or artificial light filtered through daylight glass may be used. In neither case should the light be too strong, or the readings will be less accurate. If both halves of the field do not match exactly, the position of the instrument may be shifted slightly in an attempt to make them match. If a match is still not obtained, something differs in the optics of the two sides. The most common source of error in this respect is bubbles under the plungers.

5. After the colorimeter is correctly adjusted with respect to the light source, it should not be moved during the determinations. Empty the left hand cup and rinse it and the plunger with the unknown solution, partly fill the cup, and raise it until the plunger is immersed, avoiding bubbles under the latter. Set the left cup

at a convenient depth. usually 10, 15, or 20 mm., depending on the concentration, and adjust the depth of the right cup containing the standard, until the two halves of the field have the same color intensity. Read and record the depth. For maximum accuracy make a number of readings and average the results, but guard against eye fatigue. The advantage of setting the unknown at an even figure is that calculation is thus simplified. Concentrations of the two solutions are inversely proportional to their depth when the color intensities are equal. This relationship may be expressed by the formula

$$\text{Concentration of unknown} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{concentration of standard}$$

If, for example, in the determination of nonprotein nitrogen the standard solution contains 0.15 milligram of nitrogen in a 50 c.c. volume, and if when the unknown is set at 15 the standard reads 16.8, the amount of nitrogen in the total volume of the unknown being compared is determined as follows:

$$\text{Concentration of unknown} = \frac{16.8}{15} \times 0.15 = 0.168 \text{ mg. per 50 c.c.}$$

Results in colorimetric work are always most accurate when the unknown and the standard have nearly the same depth of color.

Where light absorption after a color-developing reaction is most intense in a particular portion of the spectrum, it is sometimes advantageous to employ light filters transmitting only this part of the spectrum. Trouble caused by the absorption of light in other parts of the spectrum by foreign substances may thus be eliminated.

PHOTO-ELECTRIC METHODS

The technic employed depends upon the type of instrument used. In general these methods depend upon the relative changes in resistance of a photo-electric cell caused by light which has passed through a standard solution, and that caused by light which has passed through the unknown solution. Manufacturers either calibrate the instruments or supply directions for their calibration. They may be used with light filters to advantage in certain cases. Although a photo-electric cell is more sensitive to changes in intensity than is the eye, the fact must be remembered that only change in intensity of total transmitted radiation is measured, and the presence in a solution of a foreign substance producing a color or a turbidity will cause erroneous results.

NEPHELOMETRIC METHODS

1. The Duboscq Colorimeter has been adapted for nephelometric determinations by Kober, Bloor and others. The Bausch and Lomb attachment shown in Figure 348 is satisfactory, likewise those furnished by the International Instrument Company of Cambridge, Mass.

2. Directions for use usually accompany the instruments.

3. As stated by Hawk, "the amounts of precipitate in solutions examined nephelometrically are not exactly inversely proportioned to the readings of the scale. When the concentration of the unknown and of the standard are within 10% of each other (or within about 20% if the readings are made at depths as great as 50-60 mm.) accurate results may however be obtained directly. If the variations
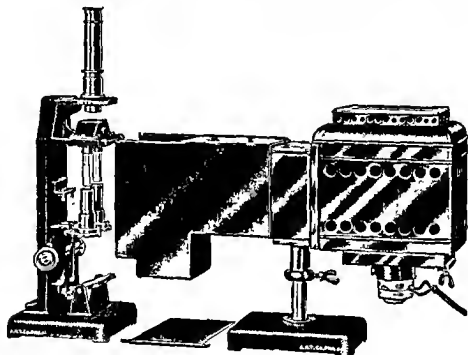
FIG. 348.—NEPHELOMETER ATTACHMENT

are greater than this a correction is necessary. Kober has proposed an equation to supply this correction and thus make possible very accurate work under conditions of moderate variations of concentration. The equation is as follows:

$$y = \frac{s}{x} - \frac{(1-x)sk}{x^2}$$

or

$$x = \frac{s + sk + \sqrt{(s+sk)^2 - 4sky}}{2y}$$

where y = height of unknown solution, on the left side of the instrument, when standard solution is kept on the right side at a definite height, s = height of standard solution on the left side and x = the ratio of the concentrations of the two solutions. $k = K/s$ where K = a constant, obtained by substitution of standardization values of s, y, and x. The instrument should be checked up for each series

of analyses by reading the standard against itself and determining the potential height of the standard solution by ·reading the scale on the left side when the solution on the right side is kept at a definite height, and the two are matched."

SCOPOMETRIC METHODS

Scopometry is performed by three different instruments: the Scopometer, the Junior Scopometer. and the Electro-Scopometer.

The Junior Scopometer (Fig. 349) provides a handy and efficient means for making many different kinds of measurements without disturbing the sample, and is unique in offering extinction and photometric criteria as well as polarization measurements in the same instrument. It is supplied by Bausch and Lomb and the filters and wedge by the Eastman Kodak Company. Full directions accompany the instrument.

Visual acuity is pooled with all other variables that might affect measurements and in practice is almost wholly eliminated. The vanishing point criterion is affected much less than the older methods by color and flocculation differences. It also allows measuring the samples in the same tubes in which the tests are made. which makes for unexampled ease and rapidity of manipulation.

The exceptional feature is the entire freedom from all troubles and inaccuracies incidental to the preparation and control of comparison standards. which it dispenses with altogether. The measurements of cloudiness and color offered by this instrument make it possible to repeat measurements of a sample without respect to the lapse of time. an advantage of practical value which may be particularly helpful to enthusiasts of permanent standards.

The use of light filters makes colorimetry with the Junior Scopometer often practicable in the presence of interfering colors and tends

FIG. 349.—THE EXTON JUNIOR SCOPOMETER

(Bausch and Lomb Optical Co., Rochester, N. Y.)

to extend greatly the range of measurement. Furthermore, by measuring the same sample with selected filters, transmitting different frequencies and repeating such measurements, transformations of a fundamental chemical nature may be subjected to practically continuous observation. and it is not beyond conjecture that this may prove to be the most interesting, if not the most valuable. feature of colorimetry with the Junior Scopometer.

It might also be mentioned that the vanishing point criterion makes it possible

to simplify technic to such an extent that determinations now regarded as too cumbersome for routine work may be brought within the realm of clinical pathology.

Although the Junior Scopometer is more accurate and reliable and gives more uniform results than the so-called permanent standards or semiquantitative methods, the extinction criterion has certain disadvantages which are inherent in the nature of its physiological optics and which prevent it achieving the accuracy of the best comparison photometry, because the human eye is unable to mark the disappearance of an object as critically as it can compare the brightness (color) of two specimens. For this reason the Electro-Scopometer more recently devised by Exton has still further increased the accuracy of scopometry.

METHODS FOR USING THE ANALYTICAL BALANCE

The balance should be kept level in a place of even temperature. In manipulating the balance all movements should be carefully made so that only a click is heard when the beam is raised and lowered. Nothing should be placed on or removed from the balance unless the mechanical supports are in place to take the weight off the knife edges. Exception may be made, however, in the case of weights smaller than 1 gram, if the pan rests are raised. With beam and pan rests having separate controls, as is the case in a good balance, the left hand manipulating the wheel moving the beam also controls the pan rests by bringing pressure of the side of the little finger against the push button. For use in keeping the balance parts free from dust and chemicals there should be provided a camel's hair brush about 1 inch wide. Objects which are to be accurately weighed should not be touched with fingers but handled with crucible tongs or otherwise. The zero point should be checked before each important weighing.

Determination of Equilibrium Point: Exact Method.—The exact limits to which the pointer moves on either side of the center of the scale are observed for an unequal number of swings and the mean of each set noted; then counting the excursions to the left "minus" and those to the right "plus," half the algebraic sum, or the mean, is taken as the equilibrium point. The amplitude of swing should be about 5 divisions on either side of the center mark. The first swing should be neglected. It is good practice to read three swings to the left and two to the right. If, for example, readings are as follows:

Divisions to Left	Divisions to Right
−5	+ 5.2
−4.6	+ 4.8
−4.3	
Sum − 13.9	+ 10
Mean − 4.63	+ 5

then the equilibrium point is $\dfrac{-4.63 + 5}{2} = + 0.18$ or 0.18 of a division to the right of the center.

Weighing.—Before weighings are started the true zero point should be determined by the above method. If the displacement of the zero point from the center of the scale is greater than one scale division, the balance should be readjusted. When weighing one of two procedures can be adopted. By adjustment of the weights and rider through trial and error the equilibrium point can be made to coincide with the true zero point. In the other less time-consuming method the equilibrium point is determined when it is brought to within several divisions of the zero point and calculation is made to the zero point by the use of sensitivity values of the balance.

Sensitivity of Balance.—The sensitivity of a balance is defined as the number of scale divisions by which the zero point is displaced by an excess in weight of 1 milligram on one side or, otherwise stated, the weight required to cause 1 scale division displacement of the equilibrium point. With increasing loads the friction on the knife edges increases and the sensitivity diminishes, so that it is necessary to know the sensitivity for various loads, and determinations are made occasionally and values kept on a card in the balance case for the following loads: no load, 1, 5, 10, 20, 50 grams. In practice the sensitivity for a given load is found by determining the variation made in the equilibrium point when 1 milligram is added to the counterpoising weights, noting the number of divisions by which the equilibrium point is changed, and then calculating the weight which would produce a variation of 1 scale division. A good analytical balance of 200 grams capacity has a sensitivity of 0.0003 gram or less without load and 0.0004 gram or less with full load. As readings are easily made to at least one-quarter of a scale division, it is seen that such a balance is well capable of weighing with an accuracy of 0.0001 gram and manufacturers commonly specify such balances as having a "sensitivity" of 0.1 milligram.

Weighing—Rapid Methods.—The procedures above outlined are too time consuming for most ordinary work and there are several shorter methods by which almost equally accurate weighings can be made. It is important, however, with most of these that the analyst know his balance, particularly the sensitivity with various loads and the loss of amplitude taking place with each successive swing and any method used should be occasionally checked by the longer procedure.

With a balance where one can regulate the initial throw of the pointer almost at will, an easy method consists in determining the zero point in terms of one set of opposite consecutive extreme excursion points (e.g., 4 divisions to the left and 3 divisions to the right) and then with load in place adjusting weights so that the pointer again moves to the same two points, or points *near by* such that the difference between the opposite consecutive excursions is the same as the difference between the zero point excursion values (e.g., with excursions at the zero point of 4 to left and 3 to right, the loaded balance could be assumed to be in equilibrium at the zero point when the pointer makes excursions of 5 to the left and 4 to the right, or 5.3 to left and 4.3 to right, etc.).

If the zero point of the unloaded balance has been adjusted to coincide with that of the scale, in the final adjustment of weights the loaded balance can also be

brought to this ideal zero point by causing the exeursion of the pointer to the right to be a certain fraction of a division less than the preceding exeursion to the left, this value being previously determined as one-half the loss in amplitude occurring during one complete cyele over approximately the same range of swing.

In the method of equal swings the balance is adjusted so that the zero point is displaced to the right by half the amount of amplitude lost during one cycle. Then the weights are taken so that at the end of each swing to the right the pointer stops on the division corresponding to the starting point at the left. Any correction for known imperfect adjustment of zero point can be made by using the sensitivity values.

Weights.—Weights are always handled with the forceps. When the weighing is finished the empty places in the box and the rider position are noted and the weight recorded. This is confirmed by observation of the weights as they lie on the balance pan and may be reconfirmed by counting as weights are replaced in the box. Weights of good quality when new are accurate enough for ordinary work, but it may be desirable and for the most accurate work essential that the degree of error affecting the weights be known and be redetermined from time to time. Manufacturers of good weights allow a tolerance of ± 0.2 milligram in the 10-gram weights. In the calibration of weights, a standard 10-gram weight is best used. If a standard weight is not available, one of the 10-gram weights in the set can be chosen as a standard, and if all weighings are done with the one set of weights, no error in analytical work will be introduced by such arbitrary choice of standard.

One of the 10-milligram weights is assumed to be correct, and provisionally corrected values of all other weights are worked out in terms of it. By using the sensitivity of the balance, as explained above, the corrected weight of the other 10-milligram weight is found to be, for instance. 9.97 milligrams. If when the 20-milligram weight is placed on the left pan, and the two 10-milligram weights are on the right pan, the correction is found from the shift in the zero point to be + 0.07 milligram, the weight of the 20-milligram weight is recorded as 10.000 + 9.97 + 0.07 = 20.01 milligrams. Thus, provisionally calibrated small weights and the rider (also calibrated against the 10-milligram weight) are combined to weigh the next larger one. When all provisionally correct weights are worked out, these values are multiplied by a factor which makes the weight of the chosen standard 10-gram weight exactly 10.0000. For instance, if after calibration of all weights on the assumption that the 10-milligram weight is correct, the standard 10-gram weight is found to weigh 10.0032 grams, all values should be multiplied by the fraction

$$\frac{10.0000}{10.0032}, \text{ or } \frac{1}{1.00032}.$$

Accessories.—In the so-called "chainomatic" balance final weight additions from 50 to 0.1 milligrams are made by simple adjustment in the length of a movable gold chain and this makes for more rapid and convenient weighing than is possible with the rider system. Weighing accessories consist of a spatula, a pair of

matched watch glasses, glazed paper squares, a pair of crucible tongs, weighing bottles of several sizes, a large and a small camel's hair brush. A large feather cut to the shape of a flag on a staff is useful for transferring dry powders. When weighing is preceded by an ignition or hot drying operation, the object should be put in a desiccator while still warm, and after cooling there for twenty minutes or longer the weighing may be made. All objects must be at room temperature when weighed.

CHAPTER XXXIII

METHODS FOR THE PREPARATION OF STANDARD VOLUMETRIC SOLUTIONS

All solutions, unless otherwise specified, are to be prepared of C.P. grades of chemicals. The word water always implies distilled water.

Normal and fractional normal volumetric solutions are not very stable, therefore they must be checked by titration if they have been standing for some time. Normal solutions may retain their strength for approximately one or two months; weaker solutions are less stable. Though a fractional normal solution can be made from a normal by dilution it is always a safer procedure to check the finished product by titrating it with a fractional normal solution known to be correct.

SULPHURIC ACID SOLUTIONS

Standard Normal Sulphuric Acid.—*Principle.*—The strength of a solution of sulphuric acid, slightly stronger than normal, is determined by titration against a known amount of sodium carbonate. It is then diluted to exactly normal and the dilution confirmed by titration.

Chemicals.—Sulphuric acid H_2SO_4, Sp. Gr. 1.84.

Sodium carbonate Na_2CO_3, anhydrous, C.P. special.

Methyl orange $NaC_{14}H_{14}N_3SO_3$.

Reagents.—Methyl Orange.—Dissolve 0.1 gram of methyl orange in water and dilute to 100 c.c.

Normal Sodium Carbonate Solution.—Dry the sodium carbonate in an oven at 105° C. for 3 to 4 hours. Weigh on an analytical balance 5.300 grams; transfer quantitatively to a 100 c.c. volumetric flask; dissolve in water and dilute to the graduation with water.

Procedure.—1. In a 2 liter beaker containing about 1100 c.c. water, slowly add about 33 c.c. sulphuric acid while stirring. Cool and transfer to a 2 liter glass-stoppered bottle. Mix thoroughly. Fill a 25 c.c. buret.

2. Into a casserole pipet accurately 20 c.c. of the normal carbonate solution. Add 2 drops methyl orange reagent. Titrate with the acid solution from the buret until a faint pink color remains on stirring. Read the amount of acid solution required from the buret. Repeat the titration with another 20 c.c. of the carbonate. They should check within 0.1 c.c.

3. Calculate the dilution as follows:

N = average of titrations in c.c.

W = c.c. of water to be added to the liter volumetric flask

$W = 50 (20 - N)$

4. Place 17 c.c. of water in liter flask; add the sulphuric acid solution to the mark. Mix thoroughly. Place some of this solution in buret and titrate as before. If the solution is correct, i.e., exactly normal, 20 c.c. will exactly neutralize 20 c.c. of the carbonate. If the average is less than 19.9 c.c., redilute as before and confirm the titration. If the average is more than 20.1 c.c., add several c.c. of concentrated acid and repeat the preparation from the beginning.

Note—1. Titrations between 19.9 and 20.1 may be accepted as sufficiently accurate for routine laboratory work.

2. In filling a buret the tube below the stop cock must be perfectly filled and free from air bubbles.

N/12 Sulphuric Acid Volumetric Solution.—Fill a 100 c.c. volumetric flask with normal sulphuric acid and transfer to a one-half gallon glass-stoppered bottle. Fill the volumetric flask with 100 c.c. of water and transfer to the bottle. Fill a one liter volumetric flask with water and add to the contents of the bottle. Mix thoroughly. This solution is N/12 sulphuric acid volumetric solution. A safe procedure is to check the finished product by titration against N/12 sodium carbonate volumetric solution.

N/12 Sulphuric Acid Volumetric Solution from Concentrated Acid.—To a liter of water in a one-half gallon glass stoppered bottle add 2.3 c.c. of concentrated sulphuric acid. Mix thoroughly. Titrate the solution with N/12 sodium carbonate solution, using methyl orange indicator.

N/12 Sodium Carbonate Volumetric Solution.—Dry the anhydrous sodium carbonate in a drying oven at 105° C. for approximately 3 hours. Weigh on the analytical balance 4.417 grams and transfer to a liter volumetric flask. Dissolve in water and dilute to the graduation with the same. Mix thoroughly.

Procedure.—Titrate 20 c.c. of the carbonate solution in a casserole with the acid solution in a buret, using methyl orange solution for the indicator. The end point is a faint pink. The amount of acid solution used should be between 19.9 and 20.1 c.c.

If less than 19.9 c.c. of acid solution are required for the titration, add water using Formula No. 1.

If more than 20.1 c.c. of acid solution are required for the titration, add concentrated acid using Formula No. 2.

V = c.c. of acid solution remaining after titration.

T = c.c. of acid solution used in titrating.

D = difference in c.c. between T and 20.

Formula No. 1 $\dfrac{V \times D}{T}$ = c.c. of water to be added to acid solution.

Formula No. 2 $\dfrac{V \times D \times 0.0023}{T}$ = c.c. of concentrated sulphuric acid to be added to the acid solution.

HYDROCHLORIC ACID SOLUTIONS

Standard Normal Hydrochloric Acid Solution.—*Principle.*—The strength of a solution of hydrochloric acid, slightly stronger than normal, is determined by titration against a known amount of sodium carbonate. It is then diluted to exactly normal and the dilution confirmed by titration.

Chemicals.—Hydrochloric acid HCl, Sp. Gr. 1.18-1.19, 35%
Sodium carbonate Na_2CO_3 anhydrous C.P. special.
Methyl orange $NaC_{14}H_{14}N_3SO_3$.

Reagents.—*Methyl Orange.*—Dissolve 0.1 gram of methyl orange in water and dilute to 100 c.c.

Normal Sodium Carbonate Solution.—Dry the sodium carbonate in an oven at 105° C. for 3 to 4 hours. Weigh on an analytical balance 5.300 grams; transfer quantitatively to a 100 c.c. volumetric flask; dissolve in water and dilute to the graduation with water.

Procedure.—1. Dilute approximately 100 c.c. of concentrated hydrochloric acid with water to 1 liter. Mix thoroughly.

2. Into a casserole pipet accurately 20 c.c. of the normal carbonate solution. Add 2 drops methyl orange reagent. Titrate with the acid solution from a buret, using methyl orange solution for the indicator. The end point is a faint pink. The amount of acid solution used should be between 19.9 and 20.1 c.c.

If less than 19.9 c.c. of acid solution are required for the titration, add water using Formula No. 1.

If more than 20.1 c.c. of acid solution are required for the titration, add concentrated acid using Formula No. 2.

3. Calculate the dilution as follows:

V = c.c. of acid solution remaining after titration.
T = c.c. of acid solution used in titrating.
D = difference in c.c. between T and 20.

Formula No. 1 $\dfrac{V \times D}{T}$ = c.c. of water to be added to acid solution.

Formula No. 2 $\dfrac{V \times D \times 0.1}{T}$ = c.c. of concentrated sulphuric acid to be added to the acid solution.

Standard N/10 and N/100 Hydrochloric Acids.—N/10 and N/100 hydrochloric acids may be prepared by accurately diluting the normal with distilled water. To make an N/10 solution, dilute 1 volume of normal to 10 volumes using distilled water.

To make an N/100 solution dilute 1 volume of normal to 100 volumes using distilled water.

SODIUM HYDROXIDE SOLUTIONS

Principle.—Since sodium hydroxide contains carbonate and is not suitable for many hydroxide solutions it is desirable to make a concentrated stock solution

from which the carbonate will separate. This is diluted for use to the strength desired.

Procedure—1. Weigh approximately 1 kilogram of sodium hydroxide and place it in a 2 liter beaker. Add 1 liter distilled water and allow to dissolve with occasional stirring. Care must be exercised because of the large amount of heat evolved. When at room temperature transfer to a rubber-stoppered bottle. Allow this to stand for a few days, when the carbonate will settle to the bottom, leaving a clear supernatant liquid. This solution will contain 70 to 75 grams sodium hydroxide in each 100 c.c.

2. Pipet accurately 5 c.c. of the stock sodium hydroxide into a 1 liter volumetric flask. Dilute to mark and mix. Pipet 20 c.c. of this diluted sodium hydroxide into a 150 c.c. Erlenmeyer flask, add 1 drop of phenolphthalein, heat to boiling, and titrate to the disappearance of the pink color with exact N/10 acid.

In case a normal solution of acid is available instead of the N/10 dilute 5 c.c. of the stock sodium hydroxide to 100 c.c. and proceed with the titration as above.

3. The calculation for either way is the same:

t = c.c. acid to titrate 20 c.c. of dilute alkali,

Gm. sodium hydroxide per 100 c.c. stock sodium hydroxide solution = $4t$

4. To make any per cent sodium hydroxide solution desired:

$$\frac{\text{per cent NaOH desired} \times 100}{\text{gram NaOH per 100 c.c. stock solution}}$$
= c.c. of the stock sodium hydroxide solution to 100 c.c. with water.

For example:

10% sodium hydroxide: $\dfrac{10 \times 100}{73}$ = 13.7 c.c. stock sodium hydroxide diluted to 100 c.c.

4.5% sodium hydroxide: $\dfrac{4.5 \times 100}{73}$ = 6.2 c.c. stock sodium hydroxide diluted to 100 c.c.

N/10 Sodium Hydroxide Solution.—The sodium hydroxide should be as free as possible from carbonates; otherwise the solution will not have the same titrating value with all common indicators.

1. For each liter of N/10 sodium hydroxide desired, pipet 7.5 c.c. of the clear concentrated solution described above and dilute to 1000 c.c. with distilled water. For routine work it is convenient to make up 6 liters of solution.

2. To determine the exact strength of the solution pipet 25 c.c. of the standard N/10 hydrochloric acid solution into a 100 c.c. Erlenmeyer flask, add 2 drops of 1% alcoholic solution of phenolphthalein, heat to boiling, and titrate. Repeat the titration until the results check within 0.1 c.c.

3. To calculate the amount of **distilled water** which must be added to make it exactly N/10, calculate as follows:

$$V = \text{c.c. of hydroxide solution remaining after titration.}$$
$$T = \text{c.c. of hydroxide solution used in titrating.}$$
$$D = \text{c.c. difference between T and 25.}$$
$$\frac{V \times D}{T} = \text{c.c. of water to be added to the hydroxide solution.}$$

4. After addition of water check by titration to be certain that the solution is exactly N/10.

5. Keep in a rubber stoppered bottle.

6. Check the normality every few weeks.

N/100 Sodium Hydroxide Solution.—Dilute the N/10 standard 1:10. Titrate against N/100 hydrochloric acid solution using phenolphthalein as an indicator.

PREPARATION OF TENTH NORMAL POTASSIUM PERMANGANATE AND SODIUM OXALATE

Principle.—Tenth normal sodium oxalate is prepared by accurately weighing pure sodium oxalate, dissolving and diluting to a definite volume. The potassium permanganate solution is prepared slightly stronger, titrated against the oxalate, and finally diluted to the exact normality.

Materials.—*Chemicals.*—Potassium permanganate, $KMnO_4$. Sodium oxalate, $Na_2C_2O_4$, (Sorenson special). Sulphuric acid, H_2SO_4.

Method.—Sodium oxalate, N/10:—dry about 10 gm. of the pure sodium oxalate in a drying oven at 105° C. for 3-4 hours. Weigh accurately 6.700 gms., dissolve in water and transfer quantitatively to a liter volumetric flask. Add 30 c.c. of concentrated sulphuric acid. Cool and dilute to mark. Mix. This is exactly N/10 sodium oxalate.

Potassium permanganate, N/10:—dissolve about 3.5 gms. potassium permanganate in about 1100 c.c. water. Set aside for about one week before standardizing. Be careful that all is dissolved. Fill the buret with this solution.

Accurately pipet 20 c.c. of the N/10 sodium oxalate solution into a casserole and warm to about 75 degrees. Titrate with the potassium permanganate until a faint pink color persists as the end point. Note the amount of permanganate used. Repeat and check within 0.1 c.c.

Calculation.

$$T = \text{average titration in c.c.}$$
$$W = \text{c.c. water to be added to the volumetric flask.}$$
$$W = 50 \ (20 - T).$$

Place W c.c. water in a liter volumetric flask. Add the permanganate solution to the mark. Mix thoroughly. This should be exactly N/10 permanganate. It is well to repeat the titration as above. The titration should be 20.00 c.c.

Notes.—The permanganate after titration should be kept in a dark place. The solution may change after it has been freshly prepared but after standing the

strength is generally constant. If it is not exactly N/10 an appropriate correction factor may be used.

MODIFIED DAKIN'S SOLUTION U.S.P. XI

(Diluted Solution of Sodium Hypochlorite)

An aqueous solution of chlorine compounds of sodium containing, in each 100 c.c. not less than 0.45 gm. and not more than 0.50 gm. of NaOCl, equivalent to not less than 0.43 gm. and not more than 0.48 gm. of available Cl.

Diluted solution of sodium hypochlorite may be prepared as follows:

Solution of sodium hypochlorite . . . . 1000 c.c.

Sodium bicarbonate.

Distilled water, of each. a sufficient quantity.

Dilute the solution of sodium hypochlorite with 5000 c.c. of distilled water and add 40 c.c. of a 5% solution of sodium bicarbonate in cold distilled water and mix well. Remove about 20 c.c. of the mixture, add to it about 0.02 gm. of powdered phenolphthalein, and shake it gently for 2 minutes. If a red color appears, add more of the sodium bicarbonate solution, and test with powdered phenolphthalein as just described, repeating the procedure as often as necessary until no red color is produced. Assay the liquid and dilute it with sufficient distilled water to make the final solution contain, in each 100 c.c., 0.48 gm. of NaOCl.

Assay—Measure accurately 25 c.c. of the solution and dilute it with 25 c.c. of distilled water. Add 1 gm. of potassium iodide and 10 c.c. of acetic acid, and titrate the liberated iodine with tenth-normal sodium thiosulphate, using starch T.S. as the indicator. Each c.c. of tenth-normal sodium thiosulphate is equivalent to 0.003723 gm. of NaOCl.

Note—Preserve diluted solution of sodium hypochlorite in well-stoppered bottles, in a cool place and protected from light.

N/10 Sodium Thiosulphate Solution.—Dissolve about 26 grams of sodium thiosulphate ($Na_2S_2O_3.5H_2O$) and 0.2 gram of sodium carbonate in a 1000 c.c. of recently boiled and cooled distilled water.

Standardize the solution by titration against N/10 iodine, or against N/10 potassium dichromate by the following method:

Measure accurately 30 c.c. of tenth-normal potassium dichromate into a glass-stoppered flask and dilute it with 50 c.c. of distilled water. Add 2 gms. of potassium iodide and 5 c.c. of hydrochloric acid, stopper and allow to stand for 10 minutes.

Dilute with 100 c.c. of distilled water and titrate the liberated iodine with the sodium thiosulphate solution. When the solution has assumed a yellowish-green color, add starch Test Solution and continue with the titration to the discharge of the blue color. Calculate the normality of the sodium thiosulphate solution and, if desired, adjust exactly to tenth-normal.

This solution should be frequently restandardized.

N/10 Potassium Dichromate Solution.—Dissolve 4.9035 gms. of reagent potassium dichromate ($K_2Cr_2O_7$), which has been pulverized and dried to constant weight at 120° Centigrade, in sufficient distilled water to measure exactly 1000 c.c. at standard temperature.

Starch Test Solution.—Triturate 1 gm. of arrowroot starch with 10 c.c. of cold distilled water and pour slowly with constant stirring into 200 c.c. of boiling distilled water. Boil the mixture until a thin, translucent fluid is obtained. Allow to settle and use only the clear, supernatant liquid. Longer boiling than necessary renders the test solution less sensitive. The test solution must be freshly prepared.

PREPARATION OF "NORMAL" PHYSIOLOGICAL SALINE SOLUTION

Physiological saline solution, or normal saline, also known as 0.85% sodium chloride solution for intravenous use, must be a sterile solution containing 0.85% of sodium chloride, chemical pure, in distilled water. The solution must be free of any minute particles and foreign chemicals. For the preparation of the solution the water must be freshly distilled.

The principle of this method consists of the preparation and standardization, by means of a chemical titration, of a 13.6% sodium chloride solution which is made once a week and is known as the stock saline solution. This solution is diluted each day with fresh distilled water, 1 liter of it to 15 liters of water, thereby obtaining a 0.85% solution of sodium chloride known as the physiological saline solution. This saline solution is also titrated to determine its per cent of sodium chloride, thereby checking any error that might have been made in diluting the stock saline solution. Both stock and physiological solutions are mixed in their preparation by drawing air through them, using a filter pump, water jet form, marketed by A. H. Thomas Co. The air is washed by drawing it through soda lime, a weak sulphuric acid solution and finely distilled water. An empty flask is placed between the suction pump and the solution being mixed, as a precaution against the filter pump "back firing."

Stock and physiological solutions are filtered by means of sintered glass filters, no paper filters being used at any time. A sintered glass filter consists of a disk of porous glass sealed in the mouth of a funnel. The filter is immersed in the solution to be filtered, the end of the neck of the filter is connected by means of rubber tubing to a perforated rubber stopper in the mouth of an empty carboy in which a vacuum is created by means of the filter pump, the filtrate being drawn through the neck of the filter, the rubber tubing and into the empty carboy.

The physiological saline solution having been standardized and filtered, is bottled in Pyrex Erlenmeyer flasks. Each flask is labeled with permanently baked-in letters "Normal Saline 0.85%." The flasks are stoppered with a paper cap or hood, the skirt or side of the cap completely covering the neck of the flask. The cap is held firmly around the neck of the flask by means of 2 wire loops that are tightened by twisting, using a tool such as is used in tightening the wire on a champagne bottle. The operation of tightening the wires requires but a few seconds of time. Each cap is stamped on the top with the date that the solution was made.

The flasks are then placed in large wire baskets capable of holding eight 2 liter flasks, each flask being in a separate compartment.

Each flask of solution is then sterilized within a few hours after its preparation.

PREPARATION OF STOCK SALINE SOLUTION

A 13.6% solution of chemical pure sodium chloride and freshly distilled water is prepared once a week. Ten liters are prepared in a 5 gallon Pyrex carboy. The solution is mixed by drawing air through it as described above. Its exact sodium chloride content is determined by means of a chemical titration which will check any error in weighing or dilution.

Several solutions are required for the titration. These are called stock solutions, to differentiate them from a group of similar solutions but of a weaker concentration used in titrating the standard saline solution.

Stock Silver Nitrate Solution.—Weigh on an analytical balance 39.529 grams of silver nitrate, dissolve in distilled water and dilute to a volume of 100 c.c. Keep in a glass-stoppered brown bottle.

Stock Ammonium Thiocyanate Solution.—180 grams of ammonium thiocyanate are dissolved in distilled water and diluted to a volume of 1 liter. This solution must be standardized against the stock silver nitrate solution, so that one volume of it will be equivalent to one volume of the silver solution. This is accomplished by pipeting 15 c.c. of stock silver nitrate solution into a 250 c.c. Erlenmeyer flask to which is added 15 c.c. of concentrated C.P. nitric acid, 0.3 gram of powdered ferric ammonium sulphate and approximately 30 c.c. of distilled water. A 25 c.c. buret is filled with the stock ammonium thiocyanate solution which is slowly titrated into the Erlenmeyer flask until a salmon pink end point is obtained that will persist for 15 seconds. If the buret reading is 15 c.c. the stock ammonium thiocyanate is correct. If less than 15 c.c. were used the thiocyanate solution is too concentrated and must be diluted using the formula:

$$\frac{\text{Volume Ammon. Thio.}}{\text{Titre}} \times (15 - \text{Titre}) = \text{c.c. of distilled water to be added.}$$

If the reading of the buret was greater than 15 c.c. then the stock thiocyanate solution is weak in concentration and ammonium thiocyanate must be added using the formula:

$$\frac{\text{Volume Ammon. Thio.}}{\text{Titre}} \times (\text{Titre} - 15) \times 0.18 = \text{grams of ammonium thiocyanate to be added}$$

All the reagents are now ready to titrate the stock saline solution.

Titration of Stock Saline Solution.—Into a 250 c.c. Erlenmeyer flask pipette 10 c.c. of stock saline solution, 15 c.c. of stock silver nitrate solution, 15 c.c. of concentrated C.P. nitric acid and 0.3 gram of powdered ferric ammonium sulphate. Allow the flask to stand 5 minutes in a dark place, then titrate with the stock ammonium thiocyanate solution. If the stock saline solution is of a correct concentration then 10 c.c. of it will combine with 10 c.c. of the stock silver nitrate

solution, therefore requiring 5 c.c. of the stock ammonium thiocyanate solution to bring the titration to the proper end point. If less than 5 c.c. of the thiocyanate solution were used the stock saline solution is of too strong a concentration and is diluted, using the formula:

$$\frac{\text{Volume Stock Saline Sol.}}{10} \times (5 - \text{Titre}) = \text{c.c. of distilled water to be added to the stock saline solution.}$$

If more than 5 c.c. of stock ammonium thiocyanate solution was required for the titration then the stock saline solution is weak in concentration and must be strengthened with sodium chloride using the formula:

$$\frac{\text{Volume Stock Saline Sol.}}{10} \times (\text{Titre} - 5) \times 0.136 = \text{grams of sodium chloride to be added to the stock saline solution.}$$

After the stock saline solution is found to be of the correct concentration, it is filtered through the sintered glass filters as described above and is then ready for dilution to make standard saline solution.

Preparation of Standard Solutions for Titrating 0.85% Saline Solution. —Using a liter volumetric flask transfer 1 liter of stock saline solution to a five gallon Pyrex flask carboy, then add 15 liters of freshly distilled water, and mix the solution by drawing air through it for about 20 minutes. This solution is physiological saline solution and should contain 0.85% of sodium chloride. To check any error that might have been made it is titrated with reagents similar to the ones used in titrating the stock saline solution, except that these reagents are weaker in concentration and are called "standard solutions" to differentiate them from the stock solutions mentioned above.

Standard Silver Nitrate Solution.—Weigh on an analytical balance 2.47 grams of silver nitrate C.P., dissolve in distilled water and dilute to a volume of 100 c.c. Keep in a glass-stoppered brown bottle. 1 c.c. of this solution is equivalent to 1 c.c. of 0.85% sodium chloride solution.

Standard Ammonium Thiocyanate Solution.—Dissolve 11.25 grams of ammonium thiocyanate C.P. in distilled water and dilute to a volume of 1 liter. This solution must be standardized against the standard silver nitrate solution in the same manner that the stock ammonium thiocyanate solution was standardized against the stock silver nitrate solution, i.e., 15 c.c. of standard silver nitrate are pipeted into a 250 c.c. Erlenmeyer flask to which is added 15 c.c. of concentrated nitric acid C.P. and 0.3 gram of powdered ferric ammonium sulphate. Approximately 15 c.c. of distilled water are added. The flask is mixed and kept in a dark place for five minutes. Fill a 25 c.c. buret with the standard ammonium thiocyanate solution and titrate into the flask to a salmon pink end point. If the buret reading is 15 c.c. the thiocyanate solution is correct and 1 c.c. of standard ammonium thiocyanate solution is equivalent to 1 c.c. of standard silver nitrate solution. If

less than 15 c.c. of the thiocyanate solution was used it is strong in concentration and must be diluted with distilled water using the formula:

$$\frac{\text{Volume Ammon. Thio.}}{\text{Titre}} \times (15 - \text{Titre}) = \text{c.c. distilled water to be added to standard ammonium thiocyanate solution.}$$

If more than 15 c.c. of thiocyanate solution were used, it is weak in concentration and ammonium thiocyanate must be added using the formula:

$$\frac{\text{Volume Ammon. Thio.}}{\text{Titre}} \times (\text{Titre} - 15) \times 0.01125 = \text{grams ammonium thiocyanate to be added to the standard ammonium thiocyanate solution.}$$

These standard reagents are now ready for use.

Titration of Physiological Saline Solution.—Pipet 20 c.c. of physiological saline solution into a 250 c.c. Erlenmeyer flask. add 25 c.c. of standard silver nitrate solution, 15 c.c. of concentrated nitric acid C.P. and 0.3 gram of powdered ferric ammonium sulphate. Mix and allow to stand in a dark place for 5 minutes. then titrate it with standard ammonium thiocyanate solution to a salmon pink end point. If the reading of the buret is 5 c.c. the saline is of the correct concentration. If the reading is less than 5 c.c., the saline solution is strong in concentration and must be diluted with distilled water using the formula:

$$\frac{\text{Total Volume of Saline}}{20} \times (5\text{-Titre}) \text{ c.c. of distilled water to be added to the physiological saline solution.}$$

If more than 5 c.c. of standard ammonium thiocyanate solution were used, the physiological saline solution is weak in concentration of sodium chloride and more stock saline solution must be added using the formula:

$$\frac{\text{Total Volume of Saline}}{20} \times \frac{(\text{Titre-5})}{15} = \text{c.c. of stock saline solution to be added to the physiological saline solution.}$$

If it was necessary to make any adjustment to the physiological saline solution it must be mixed by drawing air through it and again titrating to make certain that it is of the correct concentration.

The physiological saline solution is then filtered through the sintered glass filters and is ready for bottling.

Dispensing.—The physiological saline solution is dispensed by syphoning it into a volumetric flask of the desired volume and then pouring the contents into a permanently labeled flask, which has been thoroughly washed with hot water and soap and rinsed with tap water and finally, distilled water. The flasks are capped immediately in order that no dust particles may enter. The caps are held in place by means of two wire loops, one at the upper and one at the lower part of the skirt of the cap. The loops are tightened by means of a hook on the end of a twisted rod, on which a nut, in the form of a handle, is screwed. The operator holds the handle and pulls, thereby turning the rod and likewise twisting the wire

loop. Each flask must be examined before it leaves the laboratory for the sterilization room where it is autoclaved a short time after its manufacture.

The paper caps can be stamped with the date of manufacture.

This method of manufacturing physiological saline solution is efficient, scientific and inexpensive.

This 13.6% stock saline solution can be safely kept for a week or even longer as no bacteria will grow in it due to its high concentration of sodium chloride.

CHAPTER XXXIV

METHODS FOR THE CHEMICAL EXAMINATION OF THE BLOOD

PREPARATION OF GLASSWARE

1. Glassware should be washed as soon after use as possible. Wash with tap water, using a solution of soap made by dissolving a soap powder or chips in tap water. Rinse thoroughly with tap water and finally with distilled water and allow to dry.

2. A tall crock or cylinder full of tap water with a layer of cotton in the bottom should be kept near to receive soiled pipets immediately after using. To wash pipets hold them in the flowing tap water, or better, use a water suction pump attached to the faucet. Place the one end of the pipet in the rubber tube connected with the pump and the other end in a container of tap water and allow the pump to suck the water from the container through the pipet for about half a minute. Remove the pipet from the rubber tube and allow a little distilled water to run through the pipet, then put aside to dry.

3. When glass retains a cloudiness that cannot be removed by means of washing with soap, use potassium dichromate sulphuric acid cleaning solution, made as follows:

Technical potassium dichromate	5 gm.
Technical sulphuric acid	450 c.c.
Water	50 c.c.

Dissolve the dichromate in the water with heat before adding the acid.

Fill beakers and flasks with cleaning solution and place pipets in a tall cylinder full of solution and allow to remain twenty-four hours.

4. The cleaning fluid can be used repeatedly and when it appears to lose its strength add more potassium dichromate and sulphuric acid.

COLLECTION OF BLOOD

1. Blood is usually taken from a vein at the elbow with a sterile syringe, the technic being described and illustrated in Chapter XXVI. Blood may also be taken from a finger, if a micromethod of analysis is to be conducted as in blood sugar determinations. Umbilical cord blood may be secured at birth or specimens obtained from infants and young children by puncture of the external jugular veins or superior longitudinal sinus, the methods being described and illustrated in Chapter XXVI.

2. After standardization of diet and exercise, a pneumatic tourniquet is applied to the arm at a pressure sufficient to obstruct the venous but not the arterial circulation. Blood is withdrawn with a syringe and needle and transferred to a test tube.

3. If the method of analysis requires serum a rubber stopper is inserted into the test tube and the blood is allowed to clot. If whole blood or plasma is required for the analysis an anticoagulant must be present, a rubber stopper is inserted and the test tube inverted several times in order that the anticoagulant may come in contact with all the blood.

4. Sodium oxalate is the most generally used anticoagulant; an excess should be avoided.

5. **Preparation of Oxalated Tubes.**—Pipet 05 c.c. of a hot saturated sodium oxalate solution into test tubes. At low heat and with constant rotation evaporate the solution, leaving the dry sodium oxalate deposited in a finely divided state about the sides of the tubes. When cool insert a rubber stopper.

6. Blood sugar is rapidly destroyed (glycolysis) on standing and specimens intended for sugar determination or sugar and other determinations should have sodium fluoride (C.P. powder) added as a preservative and anticoagulant in the proportion of 60 milligrams per 10 c.c. of blood if the determination cannot be made almost immediately. As sodium fluoride is not very soluble, it is necessary to mix thoroughly to prevent clotting. If the blood is on the point of coagulating, it is well to add oxalate also. *Fluoride should not be added to specimens intended for urea determination as results will be too low.*

The addition of thymol to the fluoride is recommended for preventing or greatly inhibiting glycolysis for several days. Chlorobenzol may be used as a preservative for specimens to be kept up to ten days or longer for sugar determinations. When possible, blood samples are examined immediately after withdrawal. However, blood may be preserved in paraffin-coated tubes at a temperature of 0° C. to 5° C. for several days with practically no change in the usually sought constituents except carbon dioxide and hydrogen ion concentration.

Fig. 350.—Tube Used in Collecting Blood for Determination of pH or Carbon Dioxide.

7. A blood sample for the determination of hydrogen ion concentration is drawn in a special manner, in a centrifuge tube or heavy-walled test tube, under paraffin oil to avoid contact with air, and without stasis in the vein (Fig 350).

8. *In the following pages whenever directed to "dilute to the mark," distilled water is to be used unless some other solution is especially mentioned. The word "water" always implies distilled water.* All solutions must be thoroughly mixed after diluting to the final volume, unless instructed to the contrary.

9. The chemicals used are all of the grade of C.P. Analyzed Chemicals, unless otherwise mentioned.

10. The table on pages 712-713 indicates the amounts of blood. serum or plasma, ordinarily required for the various determinations according to the methods indicated; it also shows the normal values.

PREPARATION OF PROTEIN-FREE FILTRATE

Haden's Modification of Folin and Wu's Method.

Principle.—The proteins of blood are completely removed by filtration following precipitation with tungstic acid, which is formed by the interaction of sodium tungstate and sulphuric acid.
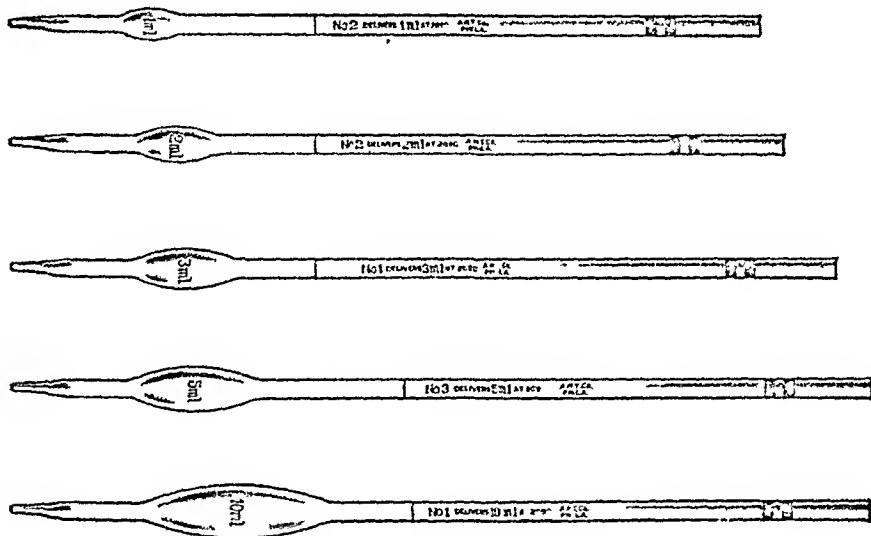
FIG. 351.—OSTWALD-FOLIN PIPETS

Materials.—Sulphuric acid, H_2SO_4. Sodium tungstate, $Na_2WO_4:2H_2O$, special according to Dr. Folin.

Reagents.—1. *Sulphuric acid N/12:* Prepared as described in Chapter XXXIII.

2. *Sodium tungstate solution:* dissolve 10 grams of sodium tungstate in water and dilute to 100 c.c.

3. The filter paper should be ammonia-free; diameters 9 to 12.5 centimeters. Whatman No. 2 is recommended.

Procedure.—1. Transfer 8 volumes of N/12 sulphuric acid to a flask with a capacity of about 125 c.c.

2. Add 1 volume of oxalated whole blood. Ostwald-Folin pipets (Fig. 351) should be used. and rinsed with the acid blood mixture several times. Allow to stand until the blood is laked as shown by the formation of brown acid hematin.

3. Add 1 volume of the sodium tungstate solution. Stopper the flask and shake thoroughly. The coagulum should be dark brown with little or no frothing.

TABLE I

Determination	Method	Normal Value	For One Determination	Blood Desired*
Sugar	Folin and Wu / Folin-McElroy	80 to 110 / 85 to 120	2 c.c. W.B. / 0.1 c.c. W.B.	5 c.c. / 0.1 c.c.
Non-protein nitrogen	Folin and Wu	25 to 35	2 c.c. W.B.	5 c.c.
Urea nitrogen†	Kerr	10 to 15	2 c.c. W.B.	5 c.c.
Uric acid	Brown	2 to 4	2 c.c. W.B.	5 c.c.
Creatinine ‡	Folin and Wu	1 to 2	3 c.c. W.B.	5 c.c.
Carbon dioxide capacity §	Van Slyke	Adults, 53 to 77 vol. per cent / Infants, 43 to 63 vol. per cent	1 c.c. P.	5 c.c.
Amino acid nitrogen	Folin	6 to 8	2 c.c. W.B.	5 c.c.
Fibrinogen	Wu	0.2 to 0.5 per cent	1 c.c. P.	6 c.c.
Albumin / Globulin / A:G ratio	Wu	16 to 6.7% / 1.2 to 2.3% / 1.5 to 2.5:1	2 c.c. S.	10 c.c.
Cholesterol	Myers & Wardell / Bloor	120 to 170 / 160 to 200	1 c.c. W.B. / 1 c.c. W.B.	3 c.c. / 3 c.c.
Total fat	Bloor	600 to 700	2 c.c. W.B.	5 c.c.
Creatine	Folin and Wu	3 to 7	1 c.c. W.B.	5 c.c.
Chlorides	Whitehorn	450 to 520	3 c.c. W.B.	3 c.c.
Calcium ‖	Clark and Collip	9 to 11	2 c.c. S.	10 c.c.
Inorganic phosphorus ¶	Fiske and Subbarow	Adults, 2.5 to 4.5 / Infants, 4 to 6	2 c.c. W.B.	5 c.c.

	Method	Normal values			
Bilirubin	Van den Bergh–McNee–Hall	0 to 0.3	2 c.c. S.	5 c.c.
Icterus index..	Meulengracht and Bernheim	4 to 6	2 c.c. S.	5 c.c.
Bromsulphthalein	Rosenthal and White	20 to 50 per cent in 5 minutes	4 c.c. S. each	8 c.c. each
Oxygen capacity ††	Van Slyke and Stadie	Men, 21 vol. per cent Women, 18 vol. per cent	2 c.c. W.B.	5 c.c.
Venous oxygen unsaturation §.	Van Slyke and Stadie	2.5 to 9.0 vol. per cent	4 c.c. W.B.	12 c.c.
Methemoglobin ††	Stadie (Spectroscopic)	0 quantitative 0 qualitative	4 c.c. W.B. 2 c.c. W.B.	10 c.c. 5 c.c.
Carbon monoxide ††	Sayers and Yant	0 to 2%	1 c.c. W.B.	5 c.c.

Normal values above refer to venous blood. Unless otherwise specified, values are expressed in milligrams per 100 c.c. W.B. = whole blood. P. = plasma. S. = serum.

* Provides excess amounts for recheck and duplicate determinations
† The micromethod may be found useful when venepuncture is difficult. As capillary blood is used, the normal values with this method may be 5 or 10 milligrams higher than normal values for venous blood. The patient's mouth should be clean and should
‡ In cases where it is impossible or impracticable to obtain a specimen of blood, urea may be determined in saliva. Send to the laboratory (about 20 c.c.) at once
be rinsed out with half a glass of water before collection is started. Send to laboratory at once
§ Collect without stasis under neutral paraffin oil in a special tube containing the necessary amount of oxalate
|| As tap water contains considerable calcium, the bloods are to be collected in syringes which have been sterilized in distilled water. Do not put in ice box.
¶ Send to laboratory at once. On standing organic phosphates break down to form inorganic phosphates and results are too high.
†† Send to the laboratory at once

4. Filter the mixture. The filtrate should be perfectly clear. Instead of filtering the mixture may be centrifuged. For the filtration of larger amounts, the Gooch crucible (Fig. 352) may be employed.

5. If the filtrate is not to be used within a short time it should be placed in the refrigerator. If it is to be kept longer than two days, a few drops of toluene should be added to prevent bacterial decomposition. Filtrates from oxalated blood may be kept overnight in the refrigerator without appreciable loss of sugar or uric acid; standing several days has little effect on the non-protein nitrogen, creatinine or creatine values.
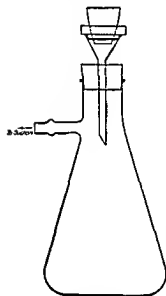
Fig. 352—FILTRATION WITH THE GOOCH CRUCIBLE

Notes.—1. If there is much foaming and the coagulum assumes a brownish-pink instead of a dark brown color, it is usually because too much oxalate is present. In such a case the sample can generally be saved by adding 10% sulphuric acid, 1 drop at a time, shaking vigorously after each drop, and continuing until there is practically no foaming and until the dark brown color has appeared.

2. If the filtrate is not clear, the precipitate and the filtrate should be returned to the flask and 10% sulphuric acid added as above to complete the protein precipitation.

3. The filtrate should be nearly neutral when the reagents are properly adjusted. With Congo red the filtrate should give a negative test and with blue litmus a positive test. Excess acidity will result in precipitation of uric acid and will give trouble in the determination of sugar by the new Folin method. To be suitable for the determination of sugar by the Folin method 10 c.c. of filtrate on titration with N/10 sodium hydroxide and 1 drop of phenolphthalein should give an endpoint with about 0.2 c.c. Haden reports 0.42 c.c. N/10 sodium hydroxide as an average titration figure for 10 c.c. filtrate.

DETERMINATION OF UREA NITROGEN

Karr's Method

Principle.—Protein-free blood filtrate is incubated with urease and phosphate buffer solution. The resultant solution is nesslerized and compared colorimetrically with a similarly treated standard urea solution.

Materials.—Two test tubes each graduated at 22.5 and 25 c.c.

Chemicals.—Gum ghatti.

    Jack bean meal.

    Alcohol C_2H_5OH, 95% U.S.P.

Metaphosphoric acid, HPO_3.

Sodium pyrophosphate, $Na_4P_2O_7 10H_2O$.

Urea $(NH_2)_2CO$.

Sodium hydroxide, NaOH.

Mercury, Hg.

Iodine, I_2.

Potassium iodide, KI.

Permutit.

Reagents.—1. *Urease.*—Place about 3 grams of permutit in a 500 c.c. flask. Add 50 c.c. of 2% acetic acid. Shake. Allow the permutit to settle, then discard the supernatant fluid. Wash the permutit twice with 50 c.c. of water, discarding each time. To the washed permutit add 15 grams of jack bean meal. Add a mixture of 16 c.c. of alcohol and 84 c.c. of water. Shake gently but continuously for 15 minutes. Allow to stand overnight in a refrigerator. When the supernatant fluid is clear, transfer it to small flasks, keeping them tightly stoppered in a refrigerator.

2. *Phosphate Buffer.*—Dissolve 14 grams of sodium pyrophosphate and 2 grams of the metaphosphoric acid in water and dilute to 250 c.c.

3. *Urea Nitrogen Stock Solution.*—Dissolve 0.1286 gram of urea in water and dilute to 200 c.c. (5 c.c. contains 1.5 milligrams urea nitrogen).

4. *Urea Nitrogen Standard Solution.*—Place 5 c.c. of the stock urea solution in a 100 c.c. volumetric flask and dilute to mark (5 c.c. contains 0.075 milligram urea nitrogen).

5. *Nessler Solution.*—See directions under Determination of Nonprotein Nitrogen, page 719.

6. *Gum Ghatti.*—Dissolve 1 gram in 100 c.c. of water. Strain through washed gauze.

Procedure.—Into an ordinary test tube marked S, pipet 5 c.c. of the standard solution of urea; into another tube marked B, pipet 5 c.c. of the protein-free blood filtrate (see page 711). Into each add 5 drops of the urease solution and 5 drops of the phosphate buffer solution. Place tubes in water bath at 50° C. for ten minutes. At the end of this time transfer quantitatively the solutions in each tube to the graduated tubes (marked S and B). Wash out the tube twice with about 5 c.c. of water into the graduated tube. Add 1 drop of 1% gum ghatti solution and dilute to the lower mark with water. Add the Nessler solution to the 25 c.c. mark. Mix by inverting. Compare in colorimeter.

Calculation:

$x =$ milligrams urea nitrogen in 100 c.c. of blood

$S =$ reading of the standard (contents of tube S)

$R =$ reading of the blood filtrate (contents of tube B)

$x = \dfrac{15S}{R}$ or $x = S$ if R is set at 15 mm.

Notes.—1. The normal range is about 10 to 15 milligrams urea nitrogen in 100 c.c. of blood.

2. The tubes in which the filtrate and standard are incubated must be kept clean and never used to contain the Nessler solution.

3. With bloods known or thought to have a high urea nitrogen content, use less filtrate and make corresponding calculation. For a large number of determinations at one time, an artificial standard may be used. The amount of urea nitrogen in the blood is affected by diet. Urea nitrogen is increased above normal in kidney insufficiency. Retention may be said to exist when the concentration reaches 20 milligrams in 100 c.c. of blood.

MICROMETHOD FOR DETERMINING UREA NITROGEN
Keller's Method

Principle.—Protein-free blood filtrate is incubated with urease and a buffer solution. The resultant solution is nesslerized and compared colorimetrically with a similarly treated standard urea solution.

Materials.—Two small test tubes each graduated at 9 and 10 c.c.

Reagents.—Same as for Karr's Method. *Tungstic Acid Solution.*—Transfer 20 c.c. of 10 per cent sodium tungstate to a liter volumetric flask. Add about 700 c.c. of water. Add, with shaking, 160 c.c. of N/12 sulphuric acid and dilute with water to 1 liter.

Procedure.—1. Place 9.8 c.c. of tungstic acid solution in a 15 c.c. centrifuge tube. Prick finger with a lancet so that the blood flows freely. Using a 0.2 c.c. serological pipet, collect 0.2 c.c. of blood. Introduce into the centrifuge tube and rinse pipet. Stopper and mix. Then centrifuge at a high velocity for a period of five minutes.

2. Into a small test tube "B" pipet 4 c.c. of the supernatant fluid. Into another small test tube "S" pipet 1 c.c. of urea nitrogen standard solution. To each tube add 3 drops of urease and 3 drops of buffer solution.

3. Digest for 10 minutes at 50° C.

4. Transfer contents to respective graduated tubes, rinsing with water and diluting to 9 c.c. mark. Add Nessler's solution to 10 c.c. mark.

5. Compare colorimetrically.

Calculation.—Set the unknown sample at 15 millimeters, when 1.25 times the reading of the standard gives milligram of urea nitrogen per 100 c.c. of blood.

DETERMINATION OF UREA NITROGEN
(Van Slyke Manometric Method)

Principles.—Alkaline hypobromite is caused to react with the urea in the tungstic acid filtrate in the reaction chamber of the Van Slyke-Neill closed type manometric apparatus and the pressure of liberated nitrogen measured at definite volume.

Materials.—Van Slyke-Neill closed type manometric apparatus (Fig. 353).

Two Ostwald-Van Slyke pipets with stop cocks, one graduated at 1 c.c., the other at 5 c.c. Both should be rubber tipped (Fig. 354).

Reagents.—1. 40% sodium hydroxide solution. 2. Bromine solution: Dissolve 60 gms. potassium bromide in 100 c.c. water. Add 2.5 gms. liquid bromine. The resultant yellow solution keeps many months in a ground glass-stoppered bottle. Sufficient alkaline hypobromite is prepared for the day's use by mixing these two solutions in the proportions of 1.25 c.c. of bromine solution to 0.75 c.c. of sodium hydroxide.

Procedure.—1. The reaction chamber is filled with mercury by raising the leveling bulb to the upper position, opening the stop cock above the reaction chamber and manipulating the stop cock leading from the leveling bulb to the reaction chamber and a small amount of mercury is allowed to run over into the cup above the chamber.

2. Using the 5 c.c. rubber-tipped pipet. the tungstic acid filtrate is drawn up to the upper mark.

3. Place the rubber-tipped end into the cup and press it firmly against the stop cock at the top of reaction chamber; open this stop cock and the one on the pipet and by lowering the mercury in the reaction chamber allow the filtrate to run into the chamber until 5 c.c. has been admitted. The flow of filtrate may be readily controlled by the stop cock with which the mercury level is controlled. Closing this stop cock stops the flow of filtrate instantly.

4. Close the stop cock on the 5 c.c. pipet and remove it from the cup.

5. Close the stop cock at the top of the reaction chamber and by lowering the leveling bulb and manipulating the lower stop cock the mercury level is brought to the 50 c.c. mark. Close the lower stop cock.

FIG. 353.—VAN SLYKE-NEILL CLOSED TYPE MANOMETRIC APPARATUS

6. Shake for one minute to remove adsorbed air.

7. Allow the mercury to flow back into the reaction chamber slowly as far as it will go. then by slowly turning the upper stop cock of the reaction chamber. permit the air bubble to escape, being careful that none of the filtrate is lost.

8. Using the 1 c.c. rubber-tipped pipet, draw up the alkaline hypobromite to the upper mark and allow it to flow into the reaction chamber in the same manner that the filtrate was introduced.

9. After closing the pipet stop cock remove it and allow a little mercury from the cup to drain into the reaction chamber, thus sealing the hole of the stop cock with mercury.

10. Draw the mercury down to the 50 c.c. mark and shake for 2 minutes.
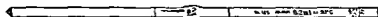
Fig. 354.—Ostwald Van Slyke Pipet

11. Allow the mercury to run back into the reaction chamber until the level of the aqueous liquid is at the 0.5 c.c. mark.

12. Read the level of the mercury in the manometer tube and the temperature of the water jacket, and then allow it to refill the tube.

13. Expel the gas from the reaction chamber by opening the stop cock at the top of the reaction chamber.

TABLE II

Factors by Which Manometer Readings Are Multiplied to Give Urea and Urea N. Content of Blood When 5 c.c. of Tungstic Acid Filtrate Is Used

Temp. °C.	Mgm. Urea N per 100 c.c. When Gas Volume Is		Mgm. Urea per 100 c.c. When Gas Volume Is	
	0.5 c.c.	2 c.c.	0.5 c.c.	2 c.c.
15	0.1561	0621	0.335	1.336
16	55	22	31	31
17	49	20	32	26
18	44	18	31	22
19	38	15	30	17
20	33	13	29	13
21	27	11	28	08
22	22	09	27	03
23	16	06	25	1.298
24	11	04	24	94
25	06	02	23	90
26	00	00	22	85
27	0.1495	0.598	21	80
28	90	96	20	76
29	85	94	19	72
30	80	92	18	67
31	74	90	16	62
32	69	88	15	58
33	64	86	14	54
34	59	84	13	50

14. Close this stop cock and bring the level of the aqueous liquid again to the 0.5 c.c. level. Read the manometer. This is the zero reading and need only be made occasionally, it being constant for any one arrangement of the apparatus.

If high urea figures are expected (this can be ascertained if the mercury in the manometer begins to rise rapidly as the liberated nitrogen is compressed in the reaction chamber), then the manometric reading is taken when the liquid level in the chamber reaches the 2 c.c. mark. From this reading is then subtracted the

manometer reading obtained after the nitrogen is expelled and the level of the liquid returned to the 2 c.c. mark.

Calculation.—The difference between the two readings is multiplied by the appropriate factor taken from the table to give mgm. of urea or urea N. per 100 c.c. of blood.

Notes.—1. The measurements, being based on direct observation of substance obtained, are independent of standard solutions. Thus it is peculiarly adapted to intermittent determinations without the necessity of checking possible deterioration of standards.

2. The simple reagents keep well and with practice a determination may be completed in 4 to 5 minutes.

3. The reaction chamber need not be cleaned between successive determinations. An occasional cleaning with dilute lactic acid (by pouring it into the cup and allowing it to drain into the reaction chamber) serves to keep the mercury free of the mercuric oxide which slowly forms. This cleaning, followed by rinsing with distilled water should be done daily if many determinations are made. Otherwise, a daily rinsing with distilled water is sufficient.

4. The usual source of error is leakage at the reaction chamber stop cock. This can be kept airtight by the use of special lubricant supplied for vacuum stop cocks.[1]

DETERMINATION OF NONPROTEIN NITROGEN

(*Folin and Wu Method*)

Principle.—The protein-free blood filtrate is treated with an acid mixture, which converts the nitrogen into ammonia. The solution is nesslerized and read against a standard ammonium sulphate similarly treated.

Materials.—One test tube, Pyrex, 75 c.c. capacity 25×200 mm. graduated at 35 and 50 c.c.

Micro burner.

Retort stand with clamp.

Chemicals.—Sulphuric acid, H_2SO_4.

Phosphoric acid, H_3PO_4 (syrupy, 85%).

Copper sulphate, $CuSO_4:5H_2O$.

Potassium iodide, KI.

Iodine, I_2.

Mercury, Hg.

Sodium hydroxide, NaOH.

Ammonium sulphate, $(NH_4)_2SO_4$ Special pyridine free.

Reagents.—1. *Acid Digestion Mixture.*—Mix 300 c.c. of C.P. syrupy phosphoric acid (85%) with 100 c.c. concentrated C.P. sulphuric acid. Transfer to a tall cylinder, cover well to exclude absorption of ammonia. and set aside for sedimentation of calcium sulphate. At the end of a week or two, pour off the supernatant fluid. To 100 c.c. of water add the 100 c.c. of the supernatant fluid and 0.6

[1] A. H. Thomas' Lubriseal has proven fairly satisfactory.

gram of copper sulphate previously dissolved in 10 c.c. of water. Ten c.c. of a 1 : 10 dilution should be neutralized by 9 to 9.3 c.c. of Nessler's reagent, phenolphthalein being used as indicator.

2. *Mercuric Potassium Iodide Solution.*—In a 500 c.c. Florence flask place 75 grams of potassium iodide, 55 grams of iodine, and 50 c.c. of water. When solution has taken place add 25 c.c. of water and 75 grams of mercury. Shake continuously until the iodine color fades. Cool the flask under running tap water, then continue shaking until the solution has a greenish color due to the formation of the double iodide. Decant the solution from the surplus mercury into a liter cylinder. Wash the residue several times with water, decanting each washing into the cylinder. Dilute the solution and washings to 1 liter. Filter if necessary.

3. 10% *Sodium Hydroxide Solution.*—Prepared as described in Chapter XXXIII.

4. *Nessler's Reagent.*—Place 350 c.c. of 10% sodium hydroxide solution in a 500 c.c. volumetric flask, add 75 c.c. of mercuric potassium iodide solution and dilute with water to the graduation. Mix. Keep in an aspirator bottle the stopper of which has a tube containing soda lime to protect the solution from carbon dioxide. Deliver the solution from the lower outlet equipped with a perforated rubber stopper, tubing and pinchcock. Discard 1 or 2 c.c. of the solution on first use each day.

Standard Ammonium Sulphate Solution.—Ammonium sulphate (C.P., special, pyridine free) should be dried in hot air for one half hour at 110° C. and then allowed to cool 20 minutes in a desiccator. Weigh on an analytical balance 0.4716 gram. Wash into a beaker to dissolve, then wash into a liter volumetric flask. Add 1 c.c. concentrated C.P. hydrochloric acid (to prevent growth of molds). Dilute to the mark with distilled water. Keep in a well-stoppered bottle labeled 3 c.c. = 0.3 milligram of nitrogen.

Notes on Nesslerization.—1. A slightly excessive concentration of alkali at once precipitates the colloidal colored ammonium compound. Local zones of excessive alkalinity occurring coagulate part of the solution. The Folin reagent is dilute enough so that localized zones of alkalinity ordinarily do not occur.

2. Turbidity in the final solution, aside from that due to silicon dioxide, is ordinarily due to the fact that the Nessler's solution is too strongly alkaline or the acid digestion mixture is too weak or too much sulphuric acid has been lost during digestion.

3. Large amounts of sulphates lead to the precipitation of the colloidal colored ammonium compound. It is for this reason that phosphoric acid is used in the digestion mixture. The most disturbing impurity is magnesium in any form and it is because of its magnesium content that tap water cannot be used for the preparation of solutions which are to be nesslerized.

4. The use of funnels is primarily to prevent loss of sulphuric acid so that the alkalinity of the nesslerized standard and unknown shall be the same. The greater the alkalinity the deeper is the color. In eliminating the loss of sulphuric acid fumes there is also eliminated all danger of losing ammonia together with those fumes.

Procedure.—1. Pipet 5 c.c. of protein-free blood filtrate (see page 711) into a dry, lipped, thin-walled, 75 c.c. Pyrex test tube (200 by 25 millimeters) graduated at 35 c.c. and 50 c.c.

2. Add 1 c.c. of the sulphuric-phosphoric acid digestion mixture and to prevent bumping add a *dry* Pyrex glass bead or a quartz pebble (may be omitted).

3. Clamp the test tube in a test tube support and boil vigorously with the micro-burner until the characteristic dense acid fumes fill the tube. This will occur in from three to seven minutes, depending on the size of the flame.

4. Turn down the flame so that the contents are just visibly boiling and close the mouth of the tube with a short funnel. Continue the heating for two minutes from the time the fumes begin to be unmistakable, even if the solution has become clear and colorless at the end of 20 to 40 seconds, as it usually does. If the oxidations are not visibly finished at the end of 2 minutes, the heating must be continued until the solution is nearly colorless.

5. Turn out the flame, remove the funnel, and allow to cool.

6. When cooled to room temperature add distilled water to the 35 c.c. mark.

7. Add Nessler's reagent to the 50 c.c. mark, insert a clean rubber stopper and mix. If the solution is turbid, centrifuge a portion, giving a crystal clear fluid above a white sediment (silica). If the sediment is colored, the nesslerization was not successful and the determination must be discarded. The unknown and the standard should be nesslerized at approximately the same time.

8. Prepare the standard as follows: Pipet 3 c.c. of ammonium sulphate standard into a 100 c.c. volumetric flask. Add 2 c.c. of the digestion mixture. Add about 50 c.c. of distilled water. Add 30 c.c. Nessler's reagent. Dilute to the mark and mix.

9. Compare the standard and unknown in the colorimeter, the standard being set at 20.

Calculation:

$$\frac{20}{R} \times 30 = \text{milligrams nonprotein nitrogen per 100 c.c. of blood.}$$

The table on page 722 shows the nonprotein nitrogen in milligrams per 100 c.c. of blood corresponding to the different colorimeter readings with 5 c.c., 2 c.c. or 1 c.c. of blood filtrate for the test.

Alternate Calculation.—When using 5 c.c. of blood filtrate, set the unknown colorimeter cup at 15 millimeters and multiply the reading of the standard cup by 2 which gives milligrams nonprotein nitrogen per 100 c.c. of blood.

Notes.—1. In the digestion, bumping is often a source of serious difficulty. The most important cause of bumping lies in the condition of the test tube. In dry test tubes and beads the very fine pores are filled with air and until this air has been driven out by heat, localized formation of steam occurs and the boiling is smooth and even, but as these pores are gradually filled with the liquid bumping begins.

2. In case of bumping after repeated determinations, heat the tube to red heat in a flame, cool and rinse with alcohol.

TABLE III

TABLE FOR NONPROTEIN NITROGEN IN BLOOD

Colorimeter Reading	Nonprotein Nitrogen in Milligrams per 100 c.c. Blood														
	Using 5 c.c of Blood Filtrate					Using 2 c.c of Blood Filtrate					Using 1 c.c of Blood Filtrate				
	.0	.2	.4	.6	.8	.0	.2	.4	.6	.8	.0	.2	.4	.6	.8
10	60.0	58.7	57.8	56.1	55.4	150.0	147.0	144.5	141.0	138.5	300	291	289	282	277
11	54.6	53.6	52.6	51.8	50.8	136.5	134.0	131.5	129.5	127.0	272	268	263	259	251
12	50.0	49.2	48.3	47.6	46.6	125.0	123.0	121.0	119.0	117.0	250	246	242	238	231
13	46.0	45.6	45.0	44.3	43.6	115.0	114.0	113.0	111.0	110.0	230	228	223	222	218
14	42.8	42.2	41.6	41.0	40.6	107.0	105.0	104.0	102.0	101.0	211	211	208	205	203
15	40.0	39.5	38.9	38.3	38.0	100.0	99.0	97.3	96.3	95.0	200	194	193	193	190
16	37.5	37.0	36.6	36.1	35.7	93.8	92.5	91.5	90.3	89.0	188	183	183	181	179
17	35.3	35.0	34.5	34.1	33.5	88.3	87.3	86.3	85.3	84.3	177	175	173	171	169
18	33.3	33.0	32.6	32.3	31.9	83.3	82.6	81.8	80.8	79.8	167	163	163	162	159
19	31.6	31.2	30.9	30.6	30.3	79.0	78.0	77.3	76.5	75.6	158	156	155	153	152
20	30.0	29.7	29.4	29.1	28.8	75.0	74.3	73.5	72.8	72.3	150	149	147	146	143
21	28.5	28.2	28.0	27.7	27.5	71.3	70.5	70.0	69.3	68.6	143	141	140	139	138
22	27.3	27.0	26.6	26.3	26.1	68.3	67.5	67.0	66.3	65.8	137	135	133	132	132
23	26.0	25.9	25.7	25.4	25.2	65.5	65.0	64.5	63.8	63.0	131	130	129	127	126
24	25.0	24.8	24.6	24.4	24.2	62.5	62.0	61.5	61.0	60.5	125	123	123	122	121
25	24.0	23.8	23.6	23.4	23.3	60.0	59.5	59.0	58.5	58.3	120	119	118	117	116
26	23.0	22.9	22.8	22.7	22.6	57.5	57.3	57.0	56.8	56.5	115	115	111	111	113
27	22.4	22.2	22.0	21.8	21.6	56.5	55.5	55.0	54.5	54.0	110	110	110	109	100
28	21.4	21.2	21.1	21.0	20.8	53.5	53.3	53.0	52.5	52.0	107	107	106	105	104
29	20.7	20.5	20.4	20.3	20.2	51.8	51.5	51.3	51.0	50.8	103	103	102	102	101

3. The amount of nonprotein nitrogen in normal blood ranges from 25 to 40 milligrams per 100 c.c.

4. The nitrogen estimated by this method represents the nitrogen of blood constituents which are not thrown down by the precipitant but remain in solution. This nitrogen has been called the "nonprotein nitrogen" and "uncoagulable nitrogen." Of the total nitrogen of the blood, it is about 1 per cent. Its principally known constituents are urea, uric acid, creatinine, creatine and amino-acids. The nitrogen in these does not equal the total nonprotein nitrogen. The difference has been called the "undetermined nitrogen" and contains principally peptid and peptone nitrogen. Urea nitrogen represents from 40 to 65% of the total, with a normal average of 50% of the total nonprotein nitrogen. Findings higher than 40 milligrams indicate nitrogenous retention; the failure of the kidney to eliminate waste products. By determining the amounts of the known constituents, particularly urea, uric acid and creatinine, more detailed information is obtained than by the determination of the total nonprotein nitrogen only.

5. In the case of bloods containing excessive amounts of nonprotein nitrogen a cloudiness results, the determination should be repeated, using 2 or 1 c.c. portions of filtrate.

DETERMINATION OF AMINO-ACIDS
(*Folin's Method*)

Principle.—The color developed by amino-acids in the presence of beta-naphtho-quinone-sulphonic acid and alkali is compared with a standard solution of an amino-acid similarly treated.

Materials.—Glycine (Ammonia-free) Eastman Kodak Co.

Sodium benzoate, C_6H_5COONa.

Hydrochloric acid, HCl.

Sodium carbonate, Na_2CO_3.

Acetic acid, CH_3COOH, 99.5%.

Sodium acetate, $CH_3COONa.3H_2O$.

Sodium thiosulphate, $Na_2S_2O_3.5H_2O$.

Sodium Beta Naphtholquinone sulphonate.

Methyl red.

Phenolphthalein.

Reagents.—1. *Stock Acid Glycine Solution.*—Make a solution of glycine (glyocoll) which will contain 0.1 milligram of nitrogen per c.c. by putting the following substances in a 500 c.c. volumetric flask:

Glycine (pure) .... 268 mg.

Sodium benzoate .... 1 gm.

N/10 hydrochloric acid q. s. ad................. 500 c.c.

This stock solution seems to keep indefinitely.

2. *Standard Acid Glycine Solution.*—Transfer 7 c.c. of stock acid glycine solution to a test tube and add 3 c.c. of N/10 hydrochloric acid solution.

3. *Special Carbonate Solution.*—Fifty c.c. of saturated sodium carbonate solution are diluted to a volume of 500 c.c. Titrate 20 c.c. of N/10 hydrochloric acid with the sodium carbonate solution using methyl red as an indicator. Dilute so that 8.5 c.c. are equivalent to 20 c.c. of the acid. The carbonate solution is about 1 per cent.

4. *Special Acetic Acid—Acetate Solution.*—Dilute 100 c.c. of 50% acetic acid with an equal volume of 5% sodium acetate.

5. *Sodium Thiosulphate Solution.*—Dissolve 4 grams of sodium thiosulphate in water and dilute to 100 c.c.

Procedure.—1. Place 1 c.c. standard acid glycine in a test tube with a capacity of 30 to 35 c.c. Label S.

2. Add 3 c.c. water.

3. In similar test tube pipet 5 c.c. of protein-free blood filtrate (see page 711). Label B.

4. Add 1 drop of 0.25% alcoholic phenolphthalein solution to each tube.

5. Add 1 c.c. special carbonate solution to standard.

6. Add special carbonate solution to filtrate drop by drop until approximately the same shade of pink is reached (3 to 4 small drops are usually required).

7. Add 5 c.c. water to standard.

8. Prepare a fresh solution of **sodium salt of beta-naphthoquinone-sulphonic acid** (100 milligrams in 20 c.c. water).

9. Add 2 c.c. of this reagent to standard and 1 c.c. to filtrate.

10. Shake a little and let stand 19 to 30 hours in a dark cupboard.

11. Add 2 c.c. of special acetic acid-acetate solution to standard. Add 1 c.c. to filtrate.

12. Add 2 c.c. thiosulphate solution to standard. Add 1 c.c. to filtrate.

13. Add 14 c.c. water to standard. Final volume equals 30 c.c. Add 7 c.c. water to filtrate. Final volume equals 15 c.c.

14. Mix and compare in colorimeter with B set at 20 mm.

Calculation:

$$X = \frac{S \times 7}{B} \text{ or } S \times 0.35 = \text{mg. amino-acid nitrogen per 100 c.c. blood.}$$

Notes.—1. The normal amino-acid content is 6 to 8 milligrams per 100 c.c. of blood.

2. Amino-acids are increased in leucemia and in acute yellow atrophy of the liver, due to tissue autolysis.

3. Insulin will reduce the amino-acid content of blood to almost as great a degree as the blood sugar.

DETERMINATION OF CREATININE

(Folin and Wu's Method)

Principle.—The yellow-red color produced in a protein-free blood filtrate by the action of alkaline picrate is compared with the color similarly produced in a standard solution of creatinine.

Materials.—Creatinine, NHC.NH.CO.CH₂.N.CH₃.
Hydrochloric acid, HCl.
Picric acid, C₆H₂OH(NO₂)₃, purified.
Sodium hydroxide, NaOH.

Reagents.—1. *Stock Creatinine Solution.*—In a 100 c.c. volumetric flask dissolve 0.1 gram of creatinine in N/10 hydrochloric acid and dilute to the mark with same.

2. *Standard Creatinine Solution.*—Pipet 3 c.c. of the stock creatinine solution into a 500 c.c. volumetric flask, add 100 c.c. of N/10 hydrochloric acid and dilute to mark with water (5 c.c. contain 0.03 milligram of creatinine).

3. *Hydrochloric Acid* (approximately N/10).—Dilute 10 c.c. of hydrochloric acid to 1 liter.

4. *Saturated Picric Acid Solution.*—Place about 15 grams of purified picric acid in a large Erlenmeyer flask; add 1 liter of water; heat over low flame until the picric acid is dissolved. Cool and keep in dark. Decant the clear solution for use.

5. *10% Sodium Hydroxide Solution.*—Prepared as described in Chapter XXXIII.

6. *Alkaline Picrate.*—To 25 c.c. of the saturated picric acid solution add 5 c.c. of sodium hydroxide solution. This should be freshly prepared for each determination.

Procedure.—1. Pipet 10 c.c. of protein-free blood filtrate (see page 711) into flask marked *B.*

2. Pipet 5 c.c. of the standard creatinine solution into a second flask, marked *S,* and add 15 c.c. of water.

3. Add 5 c.c. of the alkaline picrate solution to flask *B,* and 10 c.c. to flask *S.*

4. Mix each and let stand for ten minutes. Compare in the colorimeter.

Calculation:

$$S = \text{reading of standard}$$
$$B = \text{reading of blood filtrate}$$
$$x = \text{milligrams of creatinine per 100 c.c. of blood}$$
$$x = \frac{1.5\,S}{B} = \frac{S}{10}\text{if blood filtrate is set at 15 mm.}$$

Notes.—1. The normal range of creatinine is from 1 to 2 milligrams per 100 c.c. of blood.

2. Five c.c. of the standard gives a color for accurate colorimetric comparison with filtrates whose creatinine content is not over 2 milligrams per 100 c.c. of blood. For bloods known or thought to contain values higher than this. use 10, 15, or 20 c.c. of the standard with proportionally less water. If the content be very high, use less filtrate with proper dilution. Before the addition of the alkaline picrate the volume of *S* should be 20 c.c. and the volume of the flask *B* should be 10 c.c.

3. The saturated picric acid solution should be made from purified picric acid as described below.

Benedict's Method for Purification of Picric Acid.—Heat 6 liters of water to boiling in a large porcelain enameled pail. Add 250 grams of anhydrous sodium carbonate. When dissolved, add gradually 500 grams of moist technical picric acid. Before all of the picric acid has dissolved, the mixture should be removed from the flame and stirred a few minutes until solution of the picric acid has been effected. Filtration is usually unnecessary. Allow the solution to stand for a few minutes. Decant it from the sediment, and allow to stand overnight at room temperature. Filter with suction, using a 23 centimeter hardened filter. Suck dry. wash with 2 liters of 10% sodium chloride solution, and again suck as dry as possible. Turn off the suction. Pour 50 c.c. of diluted hydrochloric acid (1 part concentrated acid and 4 parts water) on the filter, and stir the mixture thoroughly with a porcelain spatula. This acid is then sucked through, and the process repeated with three more portions of the hydrochloric acid, a total of 2 liters. After the last portion of acid is sucked through, the picric acid on the filter is washed with 2 liters of cold distilled water and sucked dry. It is then removed from the filter and dried at about 90° C. and powdered. This product should read about 13.5 to 14 millimeters by the Folin-Doisy test.

4. The amount of creatinine is very constant for the individual and is not appreciably affected by diet, being almost entirely endogenous in origin. It is the last of the nitrogenous waste products to accumulate in nitrogenous retention. Hence its accumulation in the blood is of grave prognostic significance. In chronic conditions, once it begins to accumulate, it rarely decreases. A concentration of 5 milligrams or over per 100 c.c. of blood is usually followed by death within a short period. In acute conditions and acute exacerbations of chronic conditions, the accumulation decreases with the subsiding of the acute stage but when there has been a resulting damage to the kidney, the level will remain slightly above normal. The chief value of its determination is during the later stages of kidney disease.

DETERMINATION OF CREATINE PLUS CREATININE

Principle.—When creatine is heated with dilute acids it is transferred into its anhydride, creatinine. The creatinine preformed and that formed from creatine are then determined together by treating with alkaline picrate, as in the determination of preformed creatinine. The creatine can be calculated when the preformed creatinine is known.

Materials.—1. Same as for Creatinine Method.

Reagents.—1. *Creatinine Standard* (5 c.c. equal 0 03 milligram).—See page 724.

2. *Saturated Picric Acid Solution.*—(See page 724.)

3. 10% *Sodium Hydroxide Solution.*—Prepared as described in Chapter XXXIII.

4. *Hydrochloric Acid* (approximately N/10).—Dilute 10 c.c. of hydrochloric acid to 1 liter.

Procedure.—1. Transfer 5 c.c. of protein-free blood filtrate (see page 711) to a test tube graduated at 25 c.c. Label B.

2. Add 1 c.c. of normal hydrochloric acid.

3. Cover the mouth of the test tube with tin foil and heat in the autoclave to 130° C. for 20 minutes.

4. Cool.

5. The standard is prepared as follows: Pipet 10 c.c. of the creatinine standard into a 50 c.c. volumetric flask. Add 2 c.c. of normal hydrochloric acid. Label S.

6. Add the alkaline picrate solution (a) 5 c.c. to the unknown, (b) 10 c.c. to the standard.

7. Let stand 8 to 10 minutes.

8. With distilled water dilute (a) the unknown to the 25 c.c. mark, (b) the standard to the 50 c.c. mark.

9. Compare in the colorimeter within fifteen minutes from the time the alkaline picrate was added.

Calculation with unknown B set at 20 mm.

$$S \times 0.3 = \text{milligrams "creatinine plus creatine" per 100 c.c. of blood.}$$

Notes.—1. In the case of bloods containing large amounts of creatinine, 1, 2 or 3 c.c. of blood filtrate plus water enough to make approximately 5 c.c. are substituted for 5 c.c. of the filtrate.

2. The normal value for "total creatinine" given by this method is about 6 milligrams per 100 c.c. of blood.

DETERMINATION OF URIC ACID

(Brown's Method)

Principle.—The color produced by the action of a phosphotungstic acid reagent with the uric acid in protein-free blood filtrate is compared with the color produced by the same reagent with a standard solution of uric acid.

Materials.—Uric acid, $C_5H_4N_4O_3$.

Lithium carbonate, Li_2CO_3.

Formalin, U.S.P. (40% solution of formaldehyde).

Acetic acid, glacial, CH_3COOH.

Sodium cyanide. NaCN.

Sodium tungstate, $Na_2WO_4:2H_2O$ Special according to Dr. Folin.

Phosphoric acid. H_3PO_4, syrupy, 85%.

Ammonium hydroxide. NH_4OH.

Reagents.—1. *Stock Uric Acid Solution.*—Weigh on an analytical balance 1 gram of uric acid and transfer to a funnel on a 300 c.c. flask. Place 0.45 to 0.50 gram lithium carbonate in a beaker in about 150 c.c. of water and heat to 60° C., stirring until all the carbonate has dissolved. With the hot carbonate solution. rinse the uric acid into the flask and shake. As soon as a clear solution is obtained, cool under running water. with shaking. and transfer to a volumetric liter flask. Rinse and dilute to a volume of 400 to 500 c.c. Add 25 c.c. of formalin, and after shaking to insure thorough mixing, add 3 c.c. glacial acetic acid. Shake to remove most of the carbonic acid and dilute to the mark. Keep in small, tightly stoppered bottles in a dark place.

2. *Standard Uric Acid Solution.*—Dilute 5 c.c. of the stock solution in a liter volumetric flask to 800 c.c. with water; add 2 c.c. of formalin and 20 c.c. of two-third normal sulphuric acid. Dilute to the mark (5 c.c. are equivalent to 0.025 milligram uric acid). This keeps about eight weeks.

3. *Sodium Cyanide Solution.*—In a graduated, glass-stoppered cylinder. dissolve 10 grams of sodium cyanide. Dilute to 200 c.c. Prepare fresh about every two months.

4. *Uric Acid Reagent.*—Place in a 1000 c.c. Erlenmeyer flask. 100 grams of sodium tungstate. 80 c.c. of phosphoric acid, and about 700 c.c. of water. Boil gently for two hours using a reflux condenser. Cool and dilute to 1 liter.

Procedure.—1. Pipet 10 c.c. of protein-free blood filtrate (see page 711) into a 100 c.c. Erlenmeyer flask marked *B*: add 5 c.c. of water.

2. To two other flasks marked *SI* and *SII* add, respectively, 5 and 10 c.c. of the uric acid standard, and 10 and 5 c.c. of water.

3. To all flasks add. from buret, 5 c.c. of sodium cyanide solution. Then add 0.5 c.c. of the uric acid reagent.

4. Mix.

5. Allow to stand 20 minutes.

6. Compare in colorimeter the solution in flask B with the standard which it appears, by inspection, the more nearly to resemble in color depth.

Calculation:

$$SI = \text{reading of standard I}$$
$$SII = \text{reading of standard II}$$
$$B = \text{reading of blood filtrate}$$
$$x = \text{milligrams of uric acid in 100 c.c. of blood}$$

$$x = \frac{2.5\ SI}{B}\ \text{or}\ x = \frac{5SII}{B}$$

Set blood filtrate at 10 mm. when x $\dfrac{SI}{4}$

Set blood filtrate at 10 mm. when x $\dfrac{SII}{2}$

Notes.—1. The normal range of uric acid is from 2 to 4 milligrams per 100 c.c. of blood.

2. Uric acid is the end-product of purin metabolism, and is partly exogenous and partly endogenous.

3. It is increased in kidney disfunction but has no clinical value herewith which is not indicated by the urea nitrogen determination.

4. It is increased, with little or no increase in the other nitrogenous constituents in gout, and cardiac decompensation.

DETERMINATION OF BLOOD SUGAR
(Folin and Wu's Method)

Principle.—Protein-free blood filtrate is heated with an alkaline cupric solution. The cuprous oxide precipitate formed by the glucose is dissolved by a phosphomolybdic acid solution. The resulting solution is compared colorimetrically with one similarly prepared from a standard glucose solution.

Materials.—*Folin and Wu Sugar Tube* (Fig. 355).

Sodium tungstate $Na_2WO_4 \cdot 2H_2O$ Special according to Folin.

Sodium hydroxide, NaOH.

Phosphoric acid, H_3PO_4, U.S.P.

Molybdic acid, Anhydride, MoO_3, Special, ammonia free.

Tartaric acid, $H_2C_4H_4O_6$.

Sodium carbonate, Na_2CO_3, anhydrous.

Cupric sulphate, $CuSO_4 \cdot 5H_2O$.

Dextrose (d-Glucose) $C_6H_{12}O_6$ assayed (Bureau of Standards No. 41).

Benzoic acid, C_6H_5COOH.

Reagents.—1. *Alkaline Copper Solution.*—Dissolve 40 grams of sodium carbonate in 400 c.c. of water. Separately dissolve 7.5 grams of tartaric acid in 300 c.c. of water. Separately dissolve 4.5 grams of cupric sulphate in 200 c.c. of water. In each case use heat to hasten solution. Cool to room temperature and pour the tartaric acid solution into the carbonate solution, then add the cupric sulphate solution. Dilute to 1 liter. Mix. A red sediment may form in the course of one or two weeks. If this happens, remove the clear supernatant reagent or filter through a good quality filter paper. This reagent keeps indefinitely.

2. *Phospho-molybdic Acid Solution.*—Dissolve 40 grams of sodium hydroxide in 400 c.c. of water. Add 70 grams of molybdic acid, 10 grams of sodium tungstate and 400 c.c. of water. Boil for one-half hour. Cool. Dilute to 700 c.c. Add 250 c.c. of phosphoric acid and dilute to 1 liter. Mix.

3. *Benzoic Acid.*—Dissolve 2.5 grams of benzoic acid in 1 liter of hot water and cool. Transfer to a bottle; the solution will keep indefinitely. Filter as necessary.

Fig. 355.—Folin Blood Sugar Tube

4. *Standard Sugar Solutions.*—Stock.—Weigh 1 gram of pure dextrose (glucose) on an analytical balance. Transfer to a 100 c.c. volumetric flask, dissolve, and fill to the mark with benzoic acid solution. This 1% stock solution keeps indefinitely.

SI. Standard Containing 10 Milligrams Dextrose per 100 c.c.—Pipet 5 c.c. of stock solution into a 500 c.c. volumetric flask and dilute to the mark with benzoic acid solution.

SII. Standard Containing 20 Milligrams Dextrose per 100 c.c.—Pipet 10 c.c. of stock solution into a 500 c.c. volumetric flask and dilute to the mark with benzoic acid solution.

Standards SI and SII will keep at least 6 months.

Fig. 356.—Folin-Wu Pipet

Procedure.—1. Pipet 2 c.c. of protein-free blood filtrate (Fig. 356) into a special Folin tube (Fig. 355). Label B.

2. To a similar tube, add 2 c.c. of standard sugar solution SI. Label SI.

3. To a third Folin sugar tube add 2 c.c. of standard sugar solution SII. Label SII.

4. To each tube add 2 c.c. of the alkaline copper solution.

5. Transfer the tubes to a boiling water bath and heat for 6 minutes.

6. Cool for 2 to 3 minutes in a cold water bath without shaking.

7. Add to each tube 2 c.c. of the phospho-molybdic acid solution.

8. Let stand for 3 minutes: dilute the resulting solution to the 25 c.c. mark.

9. Insert a rubber stopper and mix. (It is essential that adequate attention be given to the mixing because the greater part of the blue color is formed in the bulb of the tube.)

10. Compare in a colorimeter with the nearest matching standard.

Calculation:

B = reading of blood sample.

$$x = \frac{100\ SI}{B} \text{ or } x = \frac{200\ SII}{B}$$

When using standard SI set the blood sample at 20 mm., when $x = 5 \times SI$.

When using standard SII set the blood sample at 10 mm., when $x = 20 \times SII$.

These results have a slight error when the standard and the unknown do not read close together. The following table is used when the *blood* sample is set and the *standard* is moved:

TABLE OF BLOOD SUGAR VALUES

Using SI set blood sample at 20 mm.				Using SII set blood sample at 10 mm.			
Reading = mg dextrose		Reading = mg dextrose		Reading = mg dextrose		Reading = mg dextrose	
5.0	30	19.5	98	6.5	142	11.4	221
5.5	32	20.0	100	7.0	150	11.6	227
6.0	35	20.5	102	7.5	157	11.8	231
6.5	37	21.0	104	8.0	166	12.0	234
7.0	40	21.5	107	8.2	169	12.5	243
7.5	42	22.0	109	8.4	173	13.0	251
8.0	45	22.5	111	8.6	176	13.5	260
8.5	47	23.0	113	8.8	180	14.0	268
9.0	50	23.5	115	9.0	183	14.5	278
9.5	52	24.0	118	9.4	190	15.0	286
10.0	55	24.5	120	9.6	193	15.5	294
10.5	57	25.0	122	9.8	197	16.0	303
11.0	60	25.5	124	10.0	200	16.5	311
11.5	63	26.0	126	10.2	203	17.0	320
12.0	65	26.5	129	10.4	207	17.5	328
12.5	67	27.0	131	10.6	210	18.0	337
13.0	69	27.5	133	10.8	214	18.5	345 *
13.5	71	28.0	135	11.0	217	19.0	354
14.0	74	28.5	137	11.2	221	19.5	363
14.5	76	29.0	139				
15.0	78	29.5	141				
15.5	80	30.0	143				
16.0	82	30.5	145				
16.5	85	31.0	146				
17.0	87	31.5	148				
17.5	89	32.0	150				
18.0	91	32.5	152				
18.5	93	33.0	154				
19.0	96	33.5	156				
		34.0	158				

Notes.—1. Cooling of the alkaline cuprous oxide suspension before adding the phosphate molybdate solution is not essential. The important point is that the standard and the unknown should not only be heated the same length of time but should also have approximately the same temperature when the acid reagent is added.

2. The normal range of concentration of blood sugar for the fasting adult is from 80 to 110 milligrams per 100 c.c. of blood.

3. If the blood filtrate gives a color too deep for accurate colorimetric comparison with the stronger standard. the test is repeated, substituting for the 2 c.c. of blood filtrate in tube *B*, 1 c.c. of blood filtrate and 1 c.c. of distilled water. The final result must therefore be doubled.

4. Dextrose determinations are made immediately after taking the blood sample as the dextrose rapidly disappears by glycolysis. Efficient refrigeration retards, but does not prevent, glycolysis.

5. When the analysis cannot be made immediately. the proteins of the blood should be precipitated and the filtrate. to which are added a few drops of toluene. placed in the refrigerator. This filtrate will give accurate readings for 24 hours after precipitation.

6. A rise in blood sugar follows absorption from the intestinal tract. The fasting level is again reached within three hours after the ingestion of food. In some pathologic·conditions, *e.g.*, diabetes mellitus. this return is delayed and the rise is higher than in normals. This forms the basis of sugar tolerance tests.

7. Hyperglycemia is found in diabetes mellitus. some cases of advanced nephritis, and frequently in emotional states.

8. Hypoglycemia has been reported after thyroidectomy and in some hypoendocrine conditions.

9. One determination of the blood sugar is not sufficient on which to base a diagnosis of hyperglycemia; the high level must be constant.

10. In "renal glycosuria" there is no hyperglycemia although there is glycosuria.

11. Glycosuria (the presence of glucose in the urine when examined by usual laboratory tests) is not dependent directly on the level of the blood sugar. The point of concentration which when reached results in glycosuria has been called the "renal threshold." This appears to be individual and not a definite point; for most normal persons concentrations of 160 to 180 milligrams result in glycosuria. These figures are higher in diabetes mellitus and late nephritis, but lower in "renal glycosuria." The significance of glycosuria cannot be determined without simultaneous blood sugar determinations.

TRUE SUGAR VALUES

The sugar content of the blood as usually determined is not chemically true as it is actually a measurement of copper reducing substances. The glucose in the blood is approximately 90% of the copper reducing material. As the physician has been taught to use normals derived from copper reducing substances it is possibly safer to retain this line of thought.

MICROMETHOD FOR THE DETERMINATION OF SUGAR

(Folin and Malmros)

Principle.—When a glucose solution is heated with an alkaline ferricyanide solution, the ferricyanide is reduced to ferrocyanide. A blue color is produced when ferrocyanide reacts with a ferric iron solution. The glucose in a protein-free filtrate is determined by comparing it with a standard glucose solution.

Materials:

Apparatus: Centrifuge tube, 15 c.c. (2).
Special Folin micro pipet, 0.1 c.c.
Test tubes, graduated at 25 c.c. (4).

Chemicals: Sodium tungstate, $Na_2WO_4:2H_2O$, "Special"
Sulphuric acid, H_2SO_4.
Potassium ferricyanide, $K_3Fe(CN)_6$.
Sodium cyanide, NaCN.
Sodium carbonate, anhydrous, Na_2CO_3.
Ferric sulphate, anhydrous, $Fe_2(SO_4)_3$.
Phosphoric acid, H_3PO_4, U.S.P.
Potassium permanganate, $KMnO_4$.
Gum ghatti.
Picric acid, $(NO_2)_3C_6H_2OH$.
Sodium hydroxide, NaOH.
Methyl alcohol, CH_3OH.
Paraffin.
Gasoline.

Reagents.—1. *Tungstic Acid Solution.*—Transfer 20 c.c. of 10% sodium tungstate to a volumetric liter flask. Dilute to a volume of 700 c.c.; add, with shaking, 160 c.c. of N/12 sulphuric acid and dilute with water to 1000 c.c.

2. *Potassium Ferricyanide Solution.*—Dissolve 2 grams of C.P. potassium ferricyanide in distilled water and dilute to a volume of 500 c.c. The major part of this solution should be kept in a brown bottle in a dark closet. The reagent in daily use should also be kept in a brown bottle.

3. *Sodium Cyanide-Carbonate Solution.*—Transfer 8 grams of anhydrous sodium carbonate to a 500 c.c. volumetric flask. Add 40 to 50 c.c. of water and shake, to promote rapid solution. Add 150 c.c. of freshly prepared 1% sodium cyanide solution; dilute to volume and mix.

4. *Ferric Iron Solution.*—Fill a liter cylinder with water. Suspend on a copper wire screen, just below the surface, 20 grams of soluble gum ghatti, and leave overnight (eighteen hours). Remove the screen, and strain the liquid through a double layer of a clean towel. Add to this extract a solution of 5 grams of anhydrous ferric sulphate in 75 c.c. of 85% phosphoric acid plus 100 c.c. of water. Add to the mixture, a little at a time, about 15 c.c. of 1% potassium permanganate solution to destroy certain reducing materials present in gum ghatti.

The slight turbidity of the solution will disappear completely, if kept at 37° C. for a few days.

5. *Standard Glucose Solution.*—To a liter volumetric flask add 1 c.c. of stock (1%) glucose solution (see page 729); add 125 c.c. of 0.2% benzoic acid; dilute to mark and mix. One c.c. = 0.01 milligram of glucose.

6. *Picrate Light Filter.*—Dissolve 5 grams of picric acid in 100 c.c. of methyl alcohol and add 5 c.c. of 10% sodium hydroxide solution. Place a pack of 8 to 10 filter papers (of the correct size to cover colorimeter lamp) on a level and smooth mat of newspapers. Pour the acid picrate solution onto the filters until
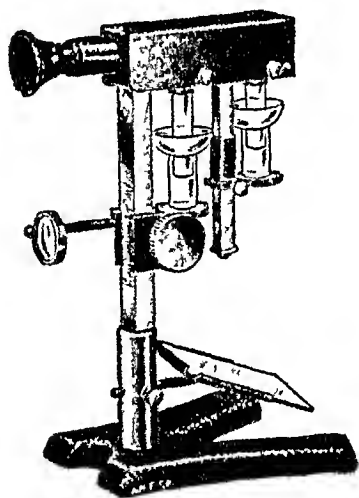
FIG. 357.—BOCK-BENEDICT COLORIMETER
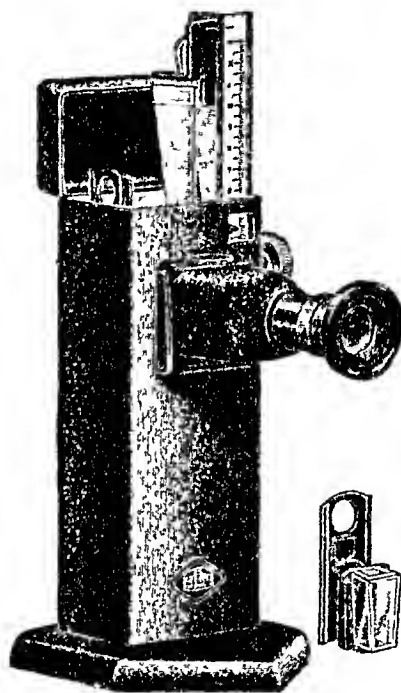
FIG. 358.—NEW HELLIGE UNIVERSAL WEDGE TYPE COLORIMETER

the papers are saturated and an excess of solution which filters through at the bottom and flows out a distance of at least 2 centimeters on the newspaper mat. When all the liquid has evaporated and the filter papers are perfectly dry, pour over the pack an excess of a 3% solution of paraffin in benzine (gasoline) and again leave the papers to dry. A heavy filter with good absorbing qualities is best.

These picrate light filters may be placed over the opening in the colorimeter light, but it is also satisfactory to cut them into pieces which may be fitted over the reflector of the colorimeter. The reflection from the yellow paper is sufficient for good color matching.

Procedure.—1. With the special 0.1 c.c. blood pipet collect 0.1 c.c. of blood from an ear or finger prick. Transfer it to 10 c.c. of dilute tungstic acid in a centrifuge tube.

2. Stir well and centrifuge or filter.

3. Transfer 4 c.c. of the supernatant fluid or filtrate to a test tube graduated at 25 c.c.

4. Transfer 4 c.c. of the standard sugar solution to a similar tube.

5. To each tube add 2 c.c. of the potassium ferricyanide solution.

6. To each add 1 c.c. of the sodium cyanide-carbonate solution.

7. Heat in boiling water for 8 minutes and cool in running water for two minutes.

8. To each add 5 c.c. of the ferric iron solution and mix.

9. Allow to stand for two minutes and dilute to the 25 c.c. graduation.

10. Using the picrate light filter, adjust the colorimeter (Figs. 357 and 358) so that the two fields have the same color intensity. Compare the two solutions.

Colorimeter readings between 40 and 5 millimeters may be accepted as correct.

Calculations:

$$x = \text{milligrams of glucose in 100 c.c. blood}$$
$$S = \text{reading of the standard}$$

Set the blood filtrate at 20 mm. when $x = 5 \times S$

Or use the table of blood sugar values given under the determination of blood sugar (see page 730).

Notes.—1. This method is recommended for use in cases where it is difficult to do a venipuncture or when the patient must be bled frequently.

2. Blood taken from the finger is a mixture of venous and arterial blood and the sugar values after a glucose meal are higher than corresponding ones from venous blood alone.

3. The picrate filters are to eliminate the yellow color of the ferricyanide solution. When the colors are nearly the same, the filter may be dispensed with.

DETERMINATION OF SUGAR TOLERANCE

1. Breakfast is omitted and the test conducted after fasting overnight.

2. Urine (No. 1) and blood (No. 1) are taken for sugar determinations.

3. Immediately thereafter give by mouth 1.75 grams of glucose per kilogram of body weight dissolved in 500 c.c. of water; flavor with lemon juice and cool with ice.

4. One hour later take urine (No. 2) and blood (No. 2) for sugar determinations.

5. One hour later (2 hours after taking the glucose), take urine (No. 3) and blood (No. 3).

6. One hour later (3 hours after taking the glucose), take urine (No. 4) and blood (No. 4).

7. As a general rule these are sufficient although it is sometimes advisable to take another specimen of urine (No. 5) and blood (No. 5) 1 hour later (4 hours after taking the glucose).

8. Conduct sugar determinations on all samples of blood and urine. Plot the blood sugar determinations in a curve.

9. Normally the blood sugar rises to about 160 milligrams per 100 c.c. at the end of the first hour, with no sugar in the urine, reaching the normal fasting level at the end of 2 to 3 hours.

10. In diabetes mellitus the blood sugar rises above 170 milligrams per 100 c.c. with sugar in the urine and does not reach the fasting level until after three or four hours.

11. In "renal glycosuria" the blood sugar rises with large amounts in the urine but declines rapidly, reaching the fasting level in three hours.

12. A sugar tolerance test should not be conducted if diabetes mellitus in an advanced state is known to be present.

One-Hour Two-Dose Sugar Tolerance Test (*Exton and Rose*).—1. Collect fasting blood and urine samples, No. 1.

2. Give patient by mouth first dose of glucose (50 grams dissolved in 325 c.c. of water). Allow one to two minutes for its ingestion.

3. Thirty minutes after ingestion of glucose, collect blood and urine samples, No. 2.

4. Give patient second dose of glucose same as above.

5. Thirty minutes after ingestion of glucose collect blood and urine samples, No. 3.

6. Make blood and urine examinations in the usual manner.

Notes.—1. This tolerance has the advantage over the five-hour older method in requiring less time and fewer samples of blood and urine. Some metabolists claim it is more specific than the older method. In the absence of endocrine disturbance it is more sensitive than the longer test for the detection of early true diabetes.

2. A normal tolerance gives a normal fasting No. 1 blood sugar and a negative No. 1 urine sugar. Blood No. 2 shows a rise not exceeding 75 milligrams and a negative No. 2 urine sugar. Blood No. 3 is less, the same or does not exceed the sugar content of No. 2 by more than 5 milligrams and a negative No. 3 urine sugar.

3. In diabetes the blood sugar of No. 3 is 10 or more milligrams higher than No. 2. The urines may or may not be positive for sugar according to the severity of the disease.

4. Should the No. 3 blood sugar rise sufficiently to classify the tolerance as being abnormal and all the urines are negative, then a fourth sample of urine may be collected one hour after No. 3 which in most cases will be positive for sugar.

DETERMINATION OF THE CARBON DIOXIDE CAPACITY OF BLOOD PLASMA

(*Van Slyke and Cullen*)

Principle.—Blood plasma is shaken in a separatory funnel filled with an air mixture the carbon dioxide tension of which approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known volume of the saturated plasma is then

run into a special apparatus (Fig. 359), acid is added and carbon dioxide is liberated by the production of a partial vacuum. The carbon dioxide is measured at atmospheric pressure and the volume corresponding to 100 c.c. of plasma is calculated.
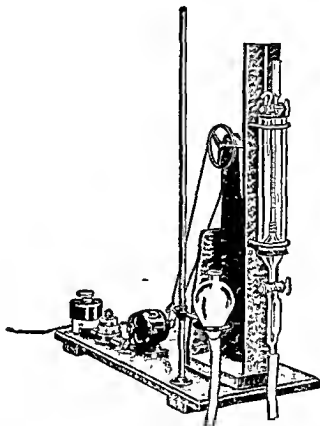
FIG 359.—PRECISION MODEL OF THE VAN SLYKE GAS ANALYSIS APPARATUS WITH WATER JACKET AND STATIC SHAKER

Materials:

    Apparatus: Van Slyke vacuum pipet.
               Barometer.
               Separatory funnel with long stem.
    Chemicals: Caprylic alcohol, $C_8H_{17}OH$.
               Lactic acid, $HC_3H_5O_3$, U.S.P.
               Mercury, Hg. redistilled.
    Solution: Lactic acid: Dilute 5 c.c. of lactic acid with water to 50 c.c.

Method.—1. Collect approximately 5 c.c. of blood from an arm vein in an oxalated test tube containing 1 c.c. of paraffin oil (Fig. 350). The stopper is loosened and the blood stirred with the inlet tube to assure mixing with the oxalate. The tube should not be shaken or inverted.

2. Remove the stopper and centrifuge the tube.

3. With a capillary pipet transfer the plasma to a separatory funnel. Putting the stem of the separatory funnel in the mouth pass the air of 3 complete exhalations through the plasma. Close the stopper and cock of the funnel and rotate it for 3 minutes.

4. Rinse the cup at the top of the Van Slyke apparatus with water, and introduce into it 1 c.c. of distilled water. Using a 1 c.c. Mohr pipet introduce beneath the water 1 c.c. of plasma. Add 1 or 2 drops of caprylic alcohol. Carefully draw the plasma, water and alcohol into the pipet until the mercury meniscus reaches the 2 c.c. mark, the upper meniscus of the solution being in the bottom of the cup of the pipet. Introduce about 1 c.c. of lactic acid solution into the cup and draw enough into the pipet to bring the mercury meniscus to the 2.5 c.c. mark. By lowering the reservoir, bring the mercury level to the 50 c.c. mark and close the lower cock. Shake for 1 minute. Slowly and without oscillation reduce the gas to atmospheric pressure by raising the surface of the mercury in the reservoir to a height equal to 1/13th of the height of the water column above the mercury in the pipet. Read the volume.

Calculation.— $X =$ cc. CO_2 bound as bicarbonate in 100 cc. plasma.

$V =$ vol. in c.c. of gas in pipet.

$B =$ observed barometric pressure in mm.

$t =$ temperature of gas, centigrade.

$$X = \frac{B}{760}(100.8 - 0.27t) \ (V + 0.002t - 0.136)$$

For barometric pressures near 760 mm. and temperatures from 20 to 25° C. an approximate calculation suitable for all clinical work is:

If V is 0.60 to 0.70 multiply by 100 and subtract 12.
If V is 0.40 to 0.60 multiply by 100 and subtract 11.
If V is 0.35 to 0.40 multiply by 100 and subtract 10.
If V is 0.25 to 0.35 multiply by 100 and subtract 9.
If V is 0.20 to 0.25 multiply by 100 and subtract 8.
If V is 0.15 to 0.20 multiply by 100 and subtract 7.
If V is 0.12 to 0.15 multiply by 100 and subtract 6.

Notes.—1. Normal range: In a normal resting adult the range is from 53 to 77 volumes per cent, and for normal infants about 10 volumes per cent lower. A result of from 53 to 40 volumes per cent shows a mild acidosis, generally without visible symptoms. In cases yielding from 40 to 31 volumes per cent symptoms may be apparent. Less than 31 volumes per cent indicates a severe acidosis.

2. When not in use, the pipet should be kept filled with distilled water.

3. Free carbonic acid is present in the body fluids in such concentration that it binds as bicarbonate all bases not bound by other acids; it therefore represents the excess of base which is left after all the nonvolatile acids have been neutralized and is available for neutralization of further acids. In this sense the bicar-

bonate constitutes the alkaline reserve of the body. Entrance of free acids reduces it to an extent proportional to the amount of the invading acids. Both in normal and pathologic metabolism, acids invade the blood and bind some of the alkali. Normally the kidneys are able to eliminate these acids while retaining the alkali; by this mechanism the body is able to excrete an acid urine from an alkaline blood.

4. Acidosis is a condition caused by acid retention sufficient to lower either the bicarbonate below normal or the pH of the blood below normal (i.e., toward the acid side). In diabetic acidosis, the acid-base balance is disturbed by abnormal formation of nonvolatile acids (e.g., aceto-aectic and beta-oxybutyric acids), while in nephritis, it is due to the failure of the kidney mechanism of elimination. In both cases the available alkali is bound by these acids reducing it in proportion to the amount of acid. Under nearly all circumstances in which the respiratory apparatus is not specifically affected, the quantity of carbonic acid is so regulated that a normal pH is maintained.

5. This method suffices for the study of such metabolic conditions as diabetes, nephritis, and marasmus, in which the acid-base disturbance is due to retention of nonvolatile acids while the respiratory control of the blood reaction is unaffected. This method is not adequate to cover conditions in which the respiratory control is so disturbed that the pH becomes abnormal, e.g., in anesthesia.

6. In such cases a determination of the hydrogen ion concentration together with the carbon dioxide content of the venous blood is of greater advantage in determining the source of the disturbance of the acid-base balance.

7. It is desirable to keep the amount of caprylic alcohol small (about 0.02 c.c.) as larger amounts may appreciably increase the results, because of the vapor tension of impurities which the alcohol may contain, and because it dissolves much more air per unit volume than does water.

8. Catch the water residue and mercury overflow in a flask. It requires only washing with water, drying with filter paper and straining through cloth or chamois skin to prepare the mercury for use again.

9. Practically the only source of difficulty with the determination is the entrance of air through the stop cocks. It is essential that both cocks should be properly greased and air tight (see below). It is also necessary that the cocks (especially the lower one) should be held in place by rubber bands so that they cannot be forced out by pressure of the mercury.

10. For thorough cleaning remove the rubber tubing and fill the apparatus by suction with aqua regia and let stand several hours.

11. If 1 c.c. of plasma is not available, 0.5 c.c. may be used, in which case the volume of distilled water and acid used is halved so that the total volume of water solution introduced is only 1.25 c.c., and in the calculation the observed volume of gas is multiplied by 2.

12. The determination can be performed on whole blood.

13. As the gas is being brought to atmospheric pressure, the meniscus of water over the mercury should be raised slowly in the narrow part of the apparatus

so that there be no oscillation of the column and resulting excessive reabsorption of carbon dioxide. With faulty technic, 0.01 to 0.02 c.c. of carbon dioxide may be reabsorbed.

14. The results of the carbon dioxide capacity determination are expressed in "volumes per cent" and have reference to the number of c.c. of carbon dioxide measured at 0° C. and 760 millimeters pressure, chemically bound as bicarbonate in 100 c.c. of blood plasma.

Stop Cock Lubricant for Van Slyke Apparatus.—One part of pure, unvulcanized, para rubber gum finely divided is dissolved by the aid of heat in 5 parts of vaselin. Keep in small ointment jars.

A thin layer of vaselin is first uniformly applied to the cock and the latter is fitted and turned several times. The rubber lubricant is then applied in the same manner. In warm weather relatively little vaselin is used; in cold weather more is needed. The two lubricants used in this manner have proved more satisfactory than a single lubricant made by dissolving the rubber gum with larger amounts of vaselin.

DETERMINATION OF CHLORIDES

(*Whitehorn*)

Principle.—The principle of the Volhard method is employed, that is, precipitation of silver chloride with silver nitrate and titration of the excess silver nitrate by means of thiocyanate. using ferric ammonium sulphate as an indicator.

Materials.—Micro buret. 5 or 10 c.c. capacity, graduated in 0.02 c.c.

Chemicals.—Silver nitrate, $AgNO_3$
Ammonium thiocyanate, NH_4CNS
Ferric ammonium sulphate, $FeNH_4(SO_4)_2.12H_2O$
Nitric acid, HNO_3, 73%.

Reagents.—*Silver Nitrate Solution.*—Weigh on an analytical balance 2.905 grams of silver nitrate. Transfer to a liter glass-stoppered volumetric flask. Dissolve in water and dilute to the graduation. Preserve in a glass-stoppered brown bottle. One c.c. is equivalent to 1 milligram of sodium chloride.

2. *Ammonium Thiocyanate Solution.*—Dissolve about 1.5 grams of ammonium thiocyanate in 1 liter of water. Standardize this solution against the silver nitrate solution by using 10 c.c. of water in place of blood filtrate and following the directions under "Procedure" given below so that 10 c.c. of the thiocyanate solution is equivalent to 10 c.c. of silver nitrate solution.

3. *Ferric Ammonium Sulphate.*—Pulverize the ferric ammonium sulphate. Weigh 3 grams of the powder and divide it into 10 approximately equal parts. Wrap each 0.3 gram of powder in a small paper.

Procedure.—1. Pipet 10 c.c. of protein-free blood filtrate into a 50 c.c. porcelain casserole.

2. Add 10 c.c. of silver nitrate solution and stir with a glass rod.

3. Add 5 c.c. of nitric acid (using a graduated cylinder) ; stir.

4. Add 0.3 gram of powdered ferric ammonium sulphate; stir.

5. Allow to stand five minutes, protected from strong light.

6. Titrate with the ammonium thiocyanate solution in the buret until a definite salmon red color persists for 15 seconds notwithstanding constant stirring.

Calculation:

t = c.c. of ammonium thiocyanate solution used.

(10 − t)100 = milligrams of sodium chloride in 100 c.c. of blood.

Notes.—1. The normal concentration of the chlorides in whole blood ranges from 450 to 520 milligrams per 100 c.c. In the plasma, the concentration ranges from 570 to 620 milligrams per 100 c.c.

2. The protein-free blood filtrate used in this method is prepared according to the method on page 711.

3. Chlorides of the blood are increased in some cases of nephritis, and in some cardiac conditions; while low values have been observed in fevers, pneumonia, severe diabetes, and after the administration of diuretics.

4. The determination of blood chlorides is of practical value as an indication or contraindication for a salt-free diet.

5. All glassware used must have been washed with distilled water and all reagents must be halogen free.

6. It is to be noted that the silver nitrate and nitric acid are not added to the protein-free filtrate simultaneously. To do so may result in the mechanical enclosure of silver nitrate solution within the curds, and a consequent error in the positive direction. The use of nitric acid is the essential point in the procedure, for aside from preventing the precipitation of silver phosphate, it flocks out the silver chlorides with a consequent reduction of the surface exposed. As silver thiocyanate is more insoluble than silver nitrate, it is evident that the surface of silver chloride exposed must be made as small as possible in order to prevent reaction between silver chloride and ferric thiocyanate. The abundance of ferric ammonium sulphate used also retards this reaction by reducing the ionization of the latter. This also deepens the end-point color by preventing the ionization of the red salt, Fe (CNS)$_3$, into yellow Fe and colorless CNS ions.

7. Mixture of tungstic acid and chloride brings down more silver than can be accounted for by chloride alone, but the possibility for error is avoided by carrying on the titration in the presence of the chloride when all the silver which has not *been precipitated by chloride is available for titration with thiocyanate.*

8. Whitehorn's greatest deviations with the method were − 1.3 and + 1.2 per cent and the limit of error with his careful technic was therefore less than 1.5%. He used volumetric flasks to make the 1:10 dilution. An error of 0.1 c.c. in measurement of silver nitrate will cause about 4 per cent error in the final result and an error of 0.1 c.c. in measurement of thiocyanate will result in about 2% error.

9. The method is applicable to plasma as well as whole blood, but loss of carbon dioxide must be prevented until plasma has been separated from corpuscles.

DETERMINATION OF INORGANIC PHOSPHORUS

(*Fiske and Subbarow*)

Principle.—The blue color obtained by adding molybdic acid and a reducing agent to an inorganic phosphate solution is compared colorimetrically with a solution similarly prepared from a standard phosphate solution.

Materials.—Ammonium molybdate, $(NH_4)_2MoO_4$.

Sulphuric acid, H_2SO_4.

Trichloracetic acid, CCl_3COOH.

Sodium bisulphite, $NaHSO_3$.

Sodium sulphite, $Na_2SO_3:7H_2O$.

Aminonaphtholsulphonic acid 1:2:4 Eastman Kodak Co.

Potassium dihydrogen phosphate, KH_2PO_4.

Reagents.—Ten times normal sulphuric acid ($10\ N\cdot H_2SO_4$) prepared as follows: 450 c.c. concentrated H_2SO_4 added to 1300 c.c. water.

Molybdate No. 1.—12.5 grams ammonium molybdate in 100 c.c. of water. To a 500-c.c. graduated cylinder add 250 c.c. $10\ N\cdot H_2SO_4$ and the molybdate solution; dilute to the mark.

Molybdate No. 2.—Prepare as above adding only 150 c.c. of acid.

10 *Per Cent Trichloracetic Acid.*—100 grams diluted to 1000 c.c. with water.

15 *Per Cent Sodium Bisulphite.*—75 grams diluted to 500 c.c. Let stand two to three days to free of turbidity. Filter. Keep well stoppered.

20 *Per Cent Sodium Sulphite.*—Dissolve 20 grams in water and dilute to 100 c.c. Filter. Keep well stoppered.

0.25 *Per Cent Aminonaphthosulphonic Acid.*—Dissolve 0.5 gram of dry powder in 195 c.c. of the sodium bisulphite solution. Add 5 c.c. of the sulphite solution. Stopper and shake until dissolved. If bisulphite is old, cloudiness results and more sulphite is required; add in 1 c.c. quantities, until clear, being careful not to add too much. This solution will keep at least two weeks.

Standard Phosphate Solution.—Dissolve 0.3509 gram of potassium dihydrogen phosphate in water and transfer quantitatively to a 1-liter volumetric flask. Add 10 c.c. of $10\ N\cdot H_2SO_4$ and dilute to the mark. Mix. Will keep indefinitely. 5 c.c. $= 0.4$ mg. P.

Blank Phosphate Correction.—Arrange three 150 c.c. beakers on a white paper. To *A*, add 100 c.c. water. To *B*, 85 c.c. water; 10 c.c. of molybdate No. 1, and 4 c.c. of sulphonic reagent. To *C*, 40 c.c. of trichloracetic acid: 45 c.c. water; 10 c.c. molybate No. 2, and 4 c.c. of sulphonic reagent. Stir. Into *B* add 1 c.c. quantities of a phosphate solution (.005 mgm. per c.c. made by diluting 5 c.c. of the standard phosphate solution to 80 c.c.) Stir after each addition and allow to stand two minutes until each have the same blue color. The number of c.c. of this phosphate multiplied by 0.05 is the correction to be subtracted from final result in analysis of blood. Solution *A* should be colorless.

Procedure.—1. To a 50 c.c. Erlenmeyer flask add 4 volumes of the trichloracetic acid solution and while gently rotating add 1 volume of oxalated blood. Stopper flask and shake vigorously and filter through an ashless filter paper.

2. Measure 5 c.c. of the filtrate into a 10 c.c. volumetric flask or graduated cylinder, add 1 c.c. of molybdate No. 2 and mix.

3. Add 0.4 c.c. of aminonapholsulphonic acid solution, dilute to 10 c.c. and mix.

4. Transfer 5 c.c. of the phosphate standard solution to a 100 c.c. volumetric flask. Add approximately 60 c.c. of water. Add 10 c.c. of molybdate No. 1 solution and 4 c.c. of aminonaphtholsulphonic acid solution. Mix after each reagent is added and dilute to the mark. Mix.

5. Compare in the colorimeter in 5 minutes.

Calculation.—When the cup of the unknown is set at 10 millimeters, the reading of the standard cup multiplied by 0.4 gives milligrams of inorganic phosphorus per 100 c.c. of blood.

Note.—1. In adults the inorganic phosphorus is about 3 milligrams in 100 c.c. of plasma. Children have a higher content, about 5 milligrams in 100 c.c. There is an increase of this phosphorus in cases of nephritis with acidosis, and a slight increase during bone repair after major fractures. In active rickets, the phosphorus is regularly reduced. In tetany, the phosphorus may be normal or reduced.

DETERMINATION OF CALCIUM
(Clark and Collip)

Principle.—The calcium precipitated as oxalate is determined by titrating the oxalic acid, liberated by acid, with standard permanganate.

Materials:

Apparatus.—Tubes, centrifuge, 15 c.c. "Pyrex."
Buret, micro, graduated in 0.02 of c.c.

Chemicals.—Ammonium oxalate, $(NH_4)_2C_2O_4$.
Sulphuric acid, H_2SO_4.
Potassium permanganate, $KMnO_4$.
Ammonium hydroxide, NH_4OH.

Reagents.—1. *Ammonium oxalate:*—Dissolve 4 gm. ammonium oxalate in water and dilute to 100 c.c.

2. *Sulphuric acid,* approximately N/1:—Dilute 28 c.c. of concentrated sulphuric acid to 1 liter.

3. *Potassium permanganate,* N/100:—Dilute 10 c.c. of exact N/10 potassium permanganate (see page 702) to 100 c.c. This solution is not sufficiently accurate for use and also changes its strength on standing. It should be titrated each day before a determination of calcium is made. Place 2 c.c. of N/100 sodium oxalate solution in a centrifuge tube. Heat it in boiling water for one minute

and titrate to the first pink color, persisting for about 15 seconds. Use the factor

$$F = \frac{2}{c.c. \text{ permanganate}} \text{ in the calculation.}$$

1. Sodium oxalate. N/100:—To a 100 c.c. vol. flask add exactly 10 c.c. of accurately standardized N/10 sodium oxalate (see page 702). Dilute to the mark with normal sulphuric acid. This solution is permanent and may be kept for standardizing the N/100 permanganate.

5. *Ammonium hydroxide:*—Dilute 2 c.c. of ammonium hydroxide to 100 c.c.

Procedure.—1. Collect about 10 c.c. of blood in a dry syringe and transfer to a plain test tube; allow it to clot: then separate serum by centrifugation.

2. If sufficient serum has been obtained run a duplicate determination.

3. Pipet 2 c.c. of water into a centrifuge tube, then 2 c.c. of serum and 1 c.c. of ammonium oxalate solution.

4. Mix thoroughly with a stirring rod.

5. Allow to stand 30 minutes.

6. Centrifuge at a high speed approximately 5 minutes. The precipitate must be well packed in the bottom of the tube.

7. Decant the supernatant liquid with care, then place the tube in a rack for 5 minutes to drain with the mouth of the tube resting on a piece of filter paper.

8. Wipe the mouth of the tube dry with a piece of filter paper.

9. With a pipet wash the sides of the tube and precipitate with 3 c.c. of the dilute ammonium hydroxide solution.

10. Centrifuge and drain as before.

11. Add 2 c.c. of the normal sulphuric acid, breaking the mat with the stirring rod.

12. Place the tube with the stirring rod in a boiling water bath for 1 minute.

13. Immediately titrate the solution with N/100 potassium permanganate solution. The end point is the faintest pink color that persists for about 1 minute.

Calculation.—T. c.c. of permanganate used in titrating serum.

$X =$ mg. of calcium per 100 c.c. of blood serum.

$F =$ Factor value of the permanganate solution.

$X = 10 \times F \times T.$

Notes.—1. The normal range is about 9 to 11 milligrams in 100 c.c. of serum. In children and infants it is slightly higher. In tetany and after parathyroidectomy. there is a decrease. Low calcium is frequently found in acute rickets. pneumonia, and in some cases of epilepsy.

2. The centrifuge tubes should be perfectly clean. They should be kept in potassium dichromate sulphuric acid cleaning solution.

3. All glassware used in the determination must be rinsed with distilled water.

4. In titrating, the permanganate should be added very slowly at the beginning, as it takes a little time for the reaction to start and oxygen will be lost if permanganic acid accumulates. The second drop should not be added until the pink color given by the first drop has disappeared. The titration temperature is impor-

tant and should be 70° to 75° C. at the start and not lower than 60° C. at the end. Otherwise too much permanganate will be used. The centrifuge tube may be conveniently held in the water bath with a test-tube holder and may be stirred by giving it a gentle whipping motion. The end-point is to be taken as the faintest persisting pink color that can be recognized when looking down the tube against a white background; at this point no pink color is recognized if one looks through the tube.

DETERMINATION OF CHOLESTEROL

(Myers and Wardell)

Principle.—The cholesterol is extracted from blood dried with plaster of Paris or washed sea sand, by continuous extraction with chloroform. The green color produced in this chloroform extract by acetic anhydride and sulphuric acid is compared with the color similarly produced in a cholesterol standard.

Materials:

Apparatus.—Soxhlet extraction apparatus, preferably syphon thimble type.
　　　　　　Mortar and pestle, glass, 3″.
　　　　　　Hot plate, electric.
　　　　　　Drying oven.

Chemicals.—Chloroform, $CHCl_3$.
　　　　　　Plaster of Paris, $2CaSO_4 \cdot H_2O$, or washed sea sand.
　　　　　　Cholesterol, $C_{27}H_{45}OH$.
　　　　　　Acetic anhydride, $(CH_3CO)_2O$.
　　　　　　Sulphuric acid, H_2SO_4.

Solutions.—Cholesterol stock: In a 100 c.c. volumetric flask, dissolve 0.16 gm. of cholesterol in chloroform and dilute to the mark with the same.

Standard cholesterol: Transfer 5 c.c. of cholesterol stock to a 100 c.c. volumetric flask and dilute to the mark with chloroform, 10 c.c. = 0.8 mg. cholesterol. (See Note 5.)

Procedure.—1. Pipet 1 c.c. of blood into about 5 grams of plaster of Paris in a glass mortar. Mix thoroughly and place in drying oven at 105° C. for one hour.

2. Transfer the pulverized dried mass to the extraction thimble. Place from 20 to 25 c.c. of chloroform in the extraction apparatus (Figs. 360 and 361) and extract for ninety minutes on the electric hot plate.

3. Transfer the chloroform extract to one of the 25 c.c. flasks, rinse and dilute to the mark with chloroform. Mix.

4. Pipet 10 c.c. of the cholesterol standard into a dry test tube. Into other dry test tubes pipet 10 c.c. of the chloroform extracts. To each add 2 c.c. of acetic anhydride and 0.2 c.c. of sulphuric acid. Place the test tubes near the colorimeter in the light by which the reading is to be made and allow to stand for fifteen minutes. Compare in the colorimeter.

Calculations:

$S =$ reading of standard extract

$R =$ reading of blood extract

$x =$ milligrams of cholesterol per 100 c.c. of blood

$$x = \frac{200S}{R}$$

Or set R at 10 millimeters when $20 \times S =$ milligrams of cholesterol per 100 c.c. of blood.

Notes.—1. The normal concentration of cholesterol in the blood ranges from 140 to 170 mg. per 100 c.c. of blood.

2. In order that the final color may be free from turbidity, all reagents must be anhydrous (chloroform and acetic anhydride redistilled). All glassware must be absolutely dry.

3. Cholesterol is partly endogenous and partly exogenous; the latter particularly from eggs, butter, meats, and some vegetables.

4. Cholesterol has been found increased in diabetes with lipemia, nephritis, complete obstruction of the common bile duct, during pregnancy and in some cases of cholethiasis and arteriosclerosis. Decreased concentration has been observed in pernicious anemia, cachexia of malignancy and in some cases of high fever.
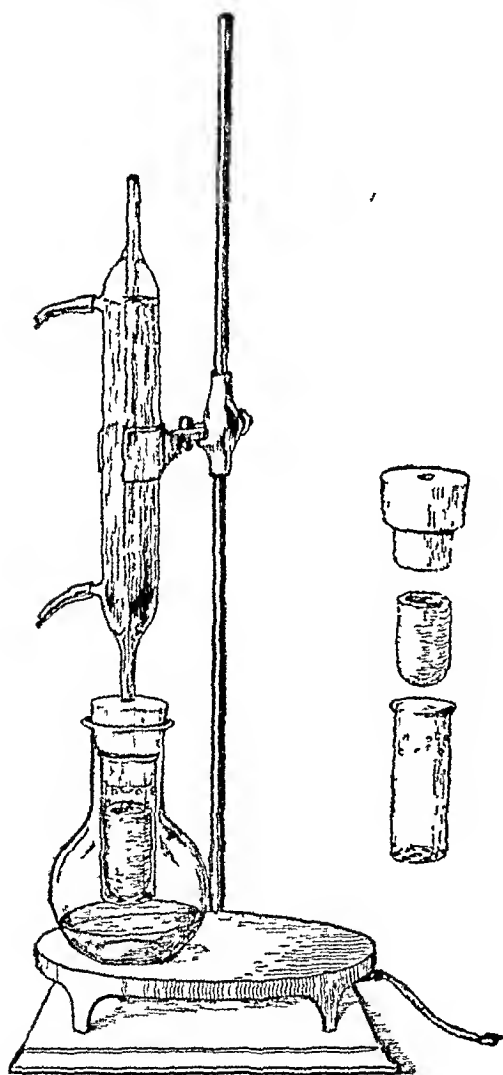
FIG. 360.—EXTRACTION APPARATUS FOR CHOLESTEROL DETERMINATION

(After Myers, *Practical Chemical Analysis of Blood*, C. V. Mosby Co.)
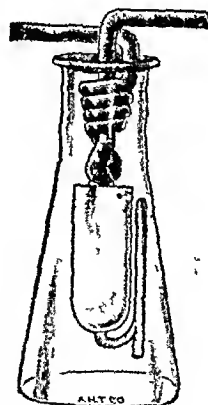
FIG. 361.—EXTRACTION APPARATUS FOR CHOLESTEROL DETERMINATION

5. An artificial color standard may be used instead of the cholesterol in chloroform solution. This consists of an 0.005% water solution of Naphthol Green B. This color should be checked against the true cholesterol standard color. Protected from light it keeps indefinitely and avoids the possibility of the standard increasing in strength due to evaporation of chloroform.

DETERMINATION OF CHOLESTEROL

(Modification of Bloor's Method)

Principle.—Cholesterol and fats are extracted from blood with an alcohol-ether solvent. After evaporation of the solvent the cholesterol is dissolved in chloroform and determined colorimetrically after the addition of acetic-anhydride and sulphuric acid.

Materials:

Alcohol, C_2H_4OH, absolute
Ether, $(C_2H_5)_2O$, anhydrous
Acid sulphuric, H_2SO_4
Acetic anhydride, $(CH_3CO)_2O$
Cholesterol, $C_{27}H_{45}OH$
Chloroform, $CHCl$

Solutions.—*Cholesterol Stock Solution.*—Weigh on an analytical balance 0.16 gram of cholesterol, transfer to a 100 c.c. volumetric flask and dilute to the graduation with chloroform. Concentration—1.6 milligrams per c.c.

Cholesterol Standard Solution.—Transfer 5 c.c. of cholesterol stock solution to a 100 c.c. volumetric flask and dilute to the mark with chloroform. Mix. Concentration—0.8 milligrams cholesterol per 10 c.c.

Alcohol-Ether Solution.—Three volumes of absolute alcohol are mixed with one volume of ether. Keep in a cool place.

Procedure.—1. Pipet 25 c.c. of alcohol-ether solution into a 50 c.c. Erlenmeyer flask. Add exactly 1 c.c. of oxalated blood, drop by drop, with agitation.

2. Boil flask for 2 minutes in a water bath.

3. Filter through Whatman paper No. 40. Rinse the flask with three 1-c.c. portions of alcohol ether solution, and pour on the filter. Collect the filtrate in a 50 c.c. beaker.

4. Evaporate the contents of the beaker into dryness, taking care that the temperature does not exceed 90° C.

5. Add 5 c.c. of chloroform to the residue, and boil 1 minute, then transfer into a 25 c.c. volumetric flask. Repeat this procedure three times with new portions of chloroform, finally diluting with chloroform to the 25 c.c. graduation. When at room temperature check the volume and mix thoroughly.

6. Pipet 10 c.c. into a test tube, label "B."

7. Pipet 10 c.c. of standard cholesterol solution into a test tube and label "S."

8. To each test tube add 2 c.c. of acetic anhydride, then 0.2 c.c. of sulphuric acid.

9. Stopper and mix.

10. Place the test tubes in a beaker of cold water and stand before the colori-
meter lamp for 15 minutes.

Calculations.—Set "B," the unknown, at 20 millimeters, when $10 \times S$ equals
milligrams of cholesterol per 100 c.c. of blood.

Notes.—The normal cholesterol with this method analysis is 160 to 200 milli-
grams per 100 c.c. of blood.

DETERMINATION OF ALBUMIN AND GLOBULIN IN BLOOD SERUM

Principle.—Total proteins are determined by using a micro Kjeldahl method
followed by nesslerization. Globulin is precipitated from blood serum by means
of sodium sulphate solution and albumin is determined in the filtrate in the
same way. A correction is made for nonprotein nitrogen. Globulin is determined
by subtracting the albumin from total protein.

Materials:

Apparatus: Incubator at 37° C.

Test tube, Pyrex, 75 c.c. 200 × 30 mm. graduated at 25 and 50 c.c.

(2). Rubber stoppers to fit.

Chemicals: Sodium chloride NaCl.

Sodium sulphate Na_2SO_4 anhydrous.

Hydrogen peroxide H_2O_2 30% "Superoxol."

Copper sulphate $CuSO_4:5H_2O$.

Sulphuric acid H_2SO_4

Ammonium sulphate $(NH_4)_2SO_4$ Special, Pyridine Free.

Mercury Hg.

Iodine I_2

Potassium Iodide KI.

Sodium hydroxide NaOH.

Reagents.—Nessler: See "Determination of Nonprotein Nitrogen in Blood,"
page 719.

Standard ammonium sulphate: See "Nonprotein Nitrogen in Blood," page 719.

Sulphuric acid digestion mixture: Dissolve 0.5 gm. of copper sulphate in 10 c.c.
of water; carefully add 100 c.c. of sulphuric acid. Pour this mixture slowly into
100 c.c. of water, stirring constantly.

Sodium sulphate solution: Weigh 22.2 gm. of anhydrous sodium sulphate and
introduce into a 100 c.c. volumetric flask. Add hot water to dissolve, cool and
dilute to the mark.

Sodium chloride solution: In a 100 c.c. volumetric flask dissolve 0.85 gm. of
sodium chloride in water and dilute to the mark.

Procedure.—1. Place in the incubator at 37° C. a 50 c.c. volumetric flask and
stopper, a 1 c.c. and a 15 c.c. volumetric pipet, two 50 c.c. stoppered cylinders,
small funnel, watch crystal, 2 filter papers 11 cm. diameter, the sodium sulphate
solution and the sodium chloride solution. Let remain for about 30 minutes.

2. The blood is collected in a plain test tube and in an oxalated test tube. The clotted sample is centrifuged and the serum placed in the incubator for 30 minutes.

3. *Total Protein Dilution:* Pipet 1 c.c. of serum into a 50 c.c. volumetric flask, add the sodium chloride solution to the mark and mix.

4. *Albumin Dilution:* Pipet 1 c.c. of serum into a 50 c.c. stoppered cylinder and using a 15 c.c. pipet, add 30 c.c. of the sodium sulphate solution. Mix. Let remain in incubator for 3 hours. Filter, using double filter papers, into a 50 c.c. cylinder, refiltering through the same papers until clear. Cover funnel with watch crystal during filtration.

5. Pipet 1 c.c. of total protein dilution into a graduated Pyrex test tube and add 1 c.c. of the sulphuric acid digestion mixture. Preferably heat in a hood using a micro burner until water has boiled off and dense white fumes appear. Continue heating slowly until solution turns black. If solution fails to clear after continued heating of one minute, add 2 drops of Superoxol and heat until solution changes to a pale straw color. Boil again one minute. Let cool. Dilute to the 25 c.c. mark.

6. Pipet 1 c.c. of the albumin dilution into a graduated Pyrex test tube and digest the same way as the total protein dilution. Dilute to the 25 c.c. mark.

7. The nitrogen content of the Superoxol must be determined. To 1 c.c. of sulphuric acid digestion mixture in a graduated Pyrex test tube slowly add 30 drops of Superoxol. Digest as above. Dilute to the 25 c.c. mark.

8. Determine the nonprotein nitrogen of the blood, using the method of "Determination of Nonprotein Nitrogen in the Blood" from the oxalated sample, see page 719.

9. *Ammonium Sulphate Standard.*—Pipet 3 c.c. of ammonium sulphate standard into a 100 c.c. volumetric flask. Add about 50 c.c. of water. Add 2 c.c. of sulphuric acid digestion mixture.

10. To each of the Pyrex test tubes add 15 c.c. of Nessler's solution. Add 30 c.c. of Nessler's solution to the standard solution. Dilute the test tubes to the 50 c.c. mark and the standard solution to the 100 c.c. mark. Stopper. Mix. Compare colorimetrically.

Calculation:

S = reading of the standard.

E = mg. nitrogen per 1 drop Superoxol.

D = number of drops Superoxol used in the digestion.

NPN = mg. nonprotein nitrogen per 100 c.c. of blood.

T = 5000 (factor for total protein dilution).

A = 3100 (factor for albumin dilution).

Set each unknown at 15 mm.

Albumin % or total protein % =

$$\left[\left(\frac{S \times 0.25}{15} - DE\right)(T \text{ or } A) - NPN\right]\frac{6.25}{1000}$$

$$E = \frac{S \times 0.15}{15 \times 30} = \text{mg. nitrogen per 1 drop Superoxol.}$$

Per cent total protein minus per cent albumin = per cent globulin

Notes: If a blood urea N. has been determined at the same time and is normal, it is not necessary to do a nonprotein nitrogen. A subtraction of 25 mg. may be used for the nonprotein nitrogen in the calculation.

It is important to separate the proteins at 37° C. and that all the solutions and glassware be at this temperature.

Frequently the digestions can be made without the use of Superoxol.

> Normal protein content of serum is 6 to 9 per cent.
> Normal albumin content of serum is 4.5 to 6.5 per cent.
> Normal globulin content of serum is 1.5 to 3 per cent.

$$\text{Protein Quotient } \frac{\text{Albumin}}{\text{Globulin}} \text{ Normal ratio is 1:1.5.}$$

In nephrosis the total protein content is decreased, as well as the albumin but the globulin is increased. Globulin is also increased in various anaphylactic conditions, in malignancy and in toxic conditions of scarlet fever, erysipelas, pneumonia and in toxemia of pregnancy.

DETERMINATION OF ALBUMIN AND GLOBULIN IN BLOOD SERUM

(*Greenberg*)

Principle.—Globulin is precipitated from blood serum by sodium sulphate solution. Albumin is determined in the filtrate by means of a phenol reagent. Total protein is determined by the same reagent. Total protein minus albumin equals globulin.

Materials.—Incubator at 37° C.
Whatman filter paper No. 42.
Sodium sulphate Na_2SO_4 anhydrous.
Sodium chloride NaCl.
Sodium hydroxide NaOH.
Tyrosine $HOC_6H_4C_2H_3(NH_2)COOH$.
Hydrochloric acid HCl.
Sodium tungstate Na_2WO_4:$2H_2O$ Special.
Sodium molybdate Na_2MoO_4:$2H_2O$.
Phosphoric acid H_3PO_4, 85 per cent.
Lithium sulphate Li_2SO_4:H_2O.
Bromine Br_2.

Reagents.—1. *Standard Tyrosine Solution.*—Using an analytical balance weigh 0.2 grams of tyrosine, transfer to a liter volumetric flask, dissolve in and dilute to the graduation using N/10 hydrochloric acid solution.

2. *N/10 Hydrochloric Acid Solution.*—For preparation see Chapter XXXIII.

3. *10 per cent Sodium Hydroxide Solution.*—For preparation see Chapter XXXIII.

4. *22.2 per cent Sodium Sulphate Solution.*—Weigh 22.2 grams of the sodium sulphate and introduce into a 100 c.c. volumetric flask. Add hot water to dissolve and dilute to the graduation.

5. *Phenol Reagent.*—Introduce into a 2 liter Pyrex Erlenmeyer flask 100 grams of sodium tungstate, 25 grams of sodium molybdate, 700 c.c. of water, 50 c.c. of phosphoric acid and 100 c.c. of concentrated hydrochloric acid. Reflux gently for 10 hours, then add 150 grams of lithium sulphate. 50 c.c. of water and a few drops of bromine. Remove the condenser and boil this mixture 15 minutes to remove excess bromine. Bromine fumes are poisonous, therefore the mixture should be boiled in a fume closet. Cool, dilute to 1 liter and filter. The solution should have no greenish tint. Preserve in a glass stoppered brown bottle.

6. *0.85 per cent Sodium Chloride Solution.*—In a 100 c.c. volumetric flask dissolve 0.85 gram of sodium chloride in water and dilute to the graduation.

7. *N/100 Sodium Hydroxide Solution.*—This solution need not be standardized; dilute 0.4 c.c. of the 10% sodium hydroxide solution to 100 c.c.

Procedure.—1. Place in an incubator at 37° C. a 1 c.c. Mohr pipet, a piece of filter paper, the sodium sulphate solution, two 6 inch test tubes and a funnel. Let remain for about 30 minutes.

2. The blood is collected in a plain test tube and allowed to clot. Centrifuge and separate the serum, placing it in the incubator for about 30 minutes.

3. Pipet 0.5 c.c. of serum into a test tube from the incubator. Add 9.5 c.c. of the sodium sulphate solution. Mix thoroughly and place in the incubator overnight.

4. Filter through the filter paper, refiltering if the filtrate is not clear. Use the filtrate for the albumin analysis. The precipitate upon the filter paper is used for the globulin analysis.

5. Albumin is determined by pipeting 5 c.c. of the filtrate into a 50 c.c. volumetric flask, adding 35 c.c. of water, 4 c.c. of the 10 per cent sodium hydroxide solution, 3 c.c. of the phenol reagent and diluting with water to the graduation. Stopper and mix.

6. A standard is prepared by pipeting 4 c.c. of the tyrosine standard solution into another 50 c.c. volumetric flask, adding 35 c.c. of water, 4 c.c. of the 10% sodium hydroxide solution, 3 c.c. of the phenol reagent and diluting with water to the graduation. Stopper and mix.

7. Globulin is determined by rinsing the test tube in which it was precipitated onto the filter paper with two 3 c.c. quantities of the sodium sulphate solution. Wash the filter paper twice with 3 c.c. quantities of the same solution. Discard the 12 c.c. of washings. Insert the neck of the funnel into a 50 c.c. volumetric flask. Perforate the filter paper and wash the precipitate into the flask, using approximately 15 or 20 c.c. of N/100 sodium hydroxide solution. Complete the washing, using approximately 10 c.c. of water. Add 4 c.c. of 10% sodium hydrox-

ide solution. 3 c.c. of the phenol reagent and dilute to the graduation with water. Stopper and mix.

8. Total protein is determined by diluting 1 c.c. of serum with 9 c.c. of the 0.85% sodium chloride solution. Mix. Pipet 2 c.c. of this dilution into another 50 c.c. volumetric flask, add 35 c.c. of water. 4 c.c. of 10% sodium hydroxide solution. 3 c.c. of the phenol reagent and dilute to the graduation with water. Stopper and mix.

9. Allow the standard and 3 unknowns to stand for 5 minutes before comparing in a colorimeter.

Calculation.—S = reading of the standard.

             B = reading of the blood serum.

             Q = the aliquot of serum used.

             F = factor for color equivalence of 1 mg. of tyrosine.

             F = 16.6 for albumin.

             F = 14.4 for globulin.

             F = 16. for total protein.

$$\frac{S \times 1 \times 100 \times F}{B \times Q \times 1000} = \text{per cent protein.}$$

or set B (albumin) at 10 mm. when $S \times 0.664$ = per cent albumin.

   set B (globulin) at 10 mm. when $S \times 0.288$ = per cent globulin.

   set B (total protein) at 10 mm. when $S \times 0.8$ = per cent total protein.

Total protein per cent minus albumin per cent equals globulin per cent.

Notes.—1. This analysis should be started the same day that the blood is collected.

2. 4 c.c. of 10 per cent sodium hydroxide is used in place of 2 c.c. of 5 times normal sodium hydroxide as required in the original method, the 10 per cent is standardized as described in Chapter XXXIII. This was done as there is very little use for the 5 times normal solution in a chemical laboratory.

3. It is not necessary to do the globulin analysis if the total protein and albumin are determined.

4. The normal protein content of serum is 6 to 9%.

   The normal albumin content of serum is 4.5 to 6.5%.

   The normal globulin content of serum is 1.0 to 3%.

DETERMINATION OF CAROTIN

Principle.—Carotin-colored serum has an orange tint while bilirubin gives it more of a straw color.

Procedure (Johnson).—1. The clear serum, practically free from corpuscles, which presses out when freshly clotted blood is allowed to clot without being disturbed. is completely desiccated with an excess of plaster of Paris.

2. The powdery mass is then moistened to a thick paste with absolute or 98% alcohol and thoroughly shaken with low boiling-point petroleum ether (30° to 50° C.).

3. Yellow discoloration of the petroleum ether signifies carotin. (Extracting pigment from alcohol-moistened serum by means of petroleum ether is equivalent to extracting the pigment from 80 to 90% alcohol by petroleum ether and indicates its carotin nature. But little carotin can be extracted from serum by direct shaking with fat solvents.)

4. The following test may also be used but is not as delicate as the above: Lipochromes, including carotin, are precipitated with proteins when 2 volumes of 95% alcohol are added to 1 volume of serum while bilirubin remains in the supernatant fluid when the precipitated proteins are centrifugalized.

DETERMINATION OF OXYGEN CAPACITY

1. The Van Slyke blood gas apparatus (see Fig. 368) is washed out twice with water before each analysis in order to remove the alkali used to absorb carbon dioxide in any previous analysis.

2. The entire apparatus is filled with mercury, including the capillaries above the upper stopcock. For 2 c.c. of blood, introduce 6 c.c. of water, 0.3 c.c. of 1 per cent saponin (Merck) solution and 2 or 3 drops of caprylic alcohol into the apparatus, and free of air by evacuation and fifteen seconds of shaking. The extracted air is expelled and the extraction is repeated until no more air is obtained.

3. The air-free solution is now drawn down and trapped in the wide branch of the apparatus below the lower stopcock. The stopcock is turned and mercury run very slowly upwards through the apparatus in order to collect the film of water left on the inside and this film is expelled through the outlet capillary on the left side. Mercury is now run into the bottom of the cup and any moisture in the cup is dried by filter paper.

4. Two c.c. of oxalated blood are now drawn into the 50 c.c. chamber from a pipet by lowering the mercury reservoir, and are trapped near the bottom of the chamber. While the upper stopcock remains open, the apparatus is shaken for two or three minutes, thus saturating the blood with oxygen.

5. The mercury is run up again into the 50 c.c. chamber, collecting the blood at the top. When the blood reaches the upper stopcock, this is closed. The lower stopcock is turned so that the previously trapped air-free water is allowed to rise in the chamber. The lower stopcock is closed and the apparatus shaken a few seconds to mix the water and blood. The blood is laked in one and one-half minutes.

6. From 0.10 to 0.12 c.c. of potassium ferricyanide solution (20 grams per 100 c.c. water) is measured into the cup and introduced into the chamber with the laked blood. (The ferricyanide may be measured with sufficient accuracy as 3 drops from a dropper which delivers 1 c.c. in 25 to 30 drops.) A mercury seal is made and the apparatus is evacuated and shaken for two to three minutes.

7. The evolved gas is composed of oxygen, nitrogen and carbon dioxide. In order to absorb the carbon dioxide the leveling bulb is placed at such a height that the mercury in it is slightly below the level of the mercury in the apparatus,

and 0.5 c.c. of 0.5 N sodium hydroxide solution (previously saturated with air by shaking) is admitted from the cup of the apparatus and allowed to trickle *slowly* down the inner wall of the chamber. If the latter part of the solution enters as a solid column instead of running down the walls, it is dislodged by letting a little mercury pass down through it in a fine stream. In any case it is usually necessary to dislodge with a drop of mercury the last drop of alkali solution which adheres just below the stopcock.

8. The fluid mixture is trapped in the bulb below the lower stopcock; mercury is run up through the left arm and the reading is made in the same manner as in the determination of carbon dioxide.

Calculation.—The volume of gas measured (V) is $O_2 + N_2 + H_2O$ and correction must be made for temperature (t) barometric pressure (B), water vapor (W) and physically dissolved air (2.1 c.c. of air being dissolved in 100 c.c. of blood at 20° C.)

$$V\left(50 \times \frac{B-W}{760(1+0.00367t)}\right) - 2.1 = \text{volume per cent oxygen capacity}$$

The value in parentheses is a factor which can be obtained from the table.

TABLE FOR THE CALCULATION OF HEMOGLOBIN CONTENT

Volumes per cent oxygen capacity×0.746 = grams hemoglobin per 100 c.c. blood. Volumes per cent oxygen capacity×4.78 = per cent hemoglobin.[*]

Temperature, Centigrade	Factor = $50 \times B - W / 760(t + 0.00367t)$
15	46.6 ×B/760
16	46.4 ×B/760
17	46.2 ×B/760
18	45.95×B/760
19	45.75×B/760
20	45.5 ×B/760
21	45.3 ×B/760
22	45.05×B/760
23	44.85×B/760
24	44.6 ×B/760
25	44.4 ×B/760
26	44.15×B/760
27	43.9 ×B/760
28	43.65×B/760
29	43.4 ×B/760
30	43.15×B/760

[*] Based on Haden's average normal of 15.6 grams.

Blood can be kept in an ice box at 60° C. for at least 24 hours before any appreciable change takes place. After a certain time blood will absorb oxygen through the oil, and the values will increase. The opposite happens when the blood is kept in the laboratory where it cannot be expected to keep constant more than two hours. After that interval the oxygen content diminished rapidly, probably on account of bacterial action. (Lundsgaard, *J. Biol. Chem.*, 1918, 33:143.)

Oxygen Unsaturation.—1. Volume per cent oxygen unsaturation equals volume per cent oxygen capacity minus volumes per cent oxygen bound by hemoglobin in venous blood.

2. This latter determination is made in the same manner as the oxygen content but the calculation is changed as follows:

$$V \left(50 \times \frac{B - W}{760(1 + 0.00367t)} \right) - 1.5 = \text{volumes per cent oxygen}$$

bound by hemoglobin in venous blood.

DETERMINATION OF METHEMOGLOBIN

Principle.—The method depends upon the fact that both hemoglobin and methemoglobin are changed quantitatively to cyanhemoglobin by dilute solutions of potassium cyanide. The color of the latter is a brilliant orange-red and is very suitable for colorimetric comparison. The change of methemoglobin to cyanhemoglobin is rapid even in the cold. Hemoglobin, however, changes slowly at room temperature, and this difficulty is avoided by converting all the hemoglobin present into methemoglobin by use of potassium ferricyanide and then converting the methemoglobin into cyanhemoglobin. The resulting solution of cyanhemoglobin is compared with a standard of known strength in a Duboscq colorimeter.

The total amount of hemoglobin plus methemoglobin having thus been determined colorimetrically, the hemoglobin content of the blood containing the two pigments (hemoglobin and methemoglobin) is determined separately from the oxygen capacity, employing the gasometric technic of Van Slyke. The hemoglobin determined by the oxygen capacity is subtracted from the hemoglobin plus methemoglobin determined together as cyanhemoglobin; the difference is methemoglobin.

Standard.—1. The hemoglobin content (grams per 100 c.c.) is determined gasometrically.

2. Then place 10 c.c. of oxalated or defibrinated blood, which is known to contain no methemoglobin, in a 500 c.c. flask.

3. Hemolyze by adding 300 c.c. water.

4. Add 2.5 c.c. of 3% potassium ferricyanide solution and let stand twenty minutes.

5. Now add 25 c.c. of 0.1% potassium cyanide solution and dilute to 500 c.c.

The blood pigment value of this solution is known from the gasometric determinations and the unknown may be compared directly with it or suitable dilutions of the standard may be made.

Example of calculation: Strength of standard equals 15 grams hemoglobin per 100 c.c. blood. Comparison of cyanhemoglobin in colorimeter: Standard 10, unknown 12. Unknown has 10/12 of 15 or 12.5 grams of total blood pigment per 100 c.c. Gasometric determination of hemoglobin equals 10 grams per 100 c.c. Therefore, sample has 12.5 less 10 or 2.5 grams of methemoglobin per 100 c.c,

Procedure.—1. Two c.c. of oxalated whole blood are placed in a 100 c.c. flask and 50 c.c. of water are added, which produces hemolysis in a few seconds.

2. Add 0.5 c.c. of a M/10 (3%) solution of potassium ferricyanide, and let stand twenty minutes.

3. Now add 5 c.c. of a 0.1% potassium cyanide solution. The change to cyanhemoglobin is immediate.

4. Water is added to the mark and the solution compared with a standard of known strength in a colorimeter. The result is the hemoglobin plus methemoglobin which is expressed as grams of "total hemoglobin pigment" per 100 cubic centimeters of blood.

5. Determine the oxygen capacity by the gasometric method of Van Slyke and multiply by 0.746 to obtain grams of hemoglobin per 100 c.c.

Calculation.—Total hemoglobin pigment per 100 c.c. minus hemoglobin per 100 c.c. which can bind oxygen equals methemoglobin per 100 c.c.

DETERMINATION OF IRON

(Wong)

Principle.—Iron is separated from hemoglobin by means of sulphuric acid and potassium persulphate. The proteins are precipitated with tungstic acid and the iron in the filtrate is determined colorimetrically by the addition of potassium thiocyanate.

Materials:

Sulphuric acid, H_2SO_4.

Potassium persulphate, $K_2S_2O_8$.

Sodium tungstate, $Na_2WO_4:2H_2O$, Special.

Potassium thiocyanate, KSCN.

Ferric ammonium sulphate, $Fe_2(SO_4)_3(NH_4)_2SO_4:24H_2$.

Acetone, $(CH_3)_2CO$.

All chemicals, except the ferric ammonium sulphate, must be iron free.

Reagents.—1. *Sodium Tungstate Solution.—*Transfer 10 grams of sodium tungstate to a 100 c.c. volumetric flask, dissolve in water and dilute to the graduation.

2. *Potassium Persulphate Solution.—*Introduce into a glass-stoppered bottle approximately 7 grams of potassium persulphate. Add approximately 100 c.c. of water. Shake well, then let stand. The excess salt will settle to the bottom. Use only the supernatant fluid.

3. *Potassium Thiocyanate Solution.—*Transfer 146 grams of potassium thiocyanate to a 500 c.c. volumetric flask. Dissolve in water and dilute to the graduation. Pour into a glass-stoppered bottle and add 20 c.c. of acetone to assist in preserving the solution.

4. *Iron Standard Solution.—*Using an analytical balance weigh 0.861 grams of ferric ammonium sulphate. Transfer to a 1 liter volumetric flask and dissolve in approximately 50 c.c. of water. Add a solution of 2 c.c. of sulphuric acid in

18 c.c. of water. Dilute with water to the 1 liter graduation. 1 c.c. is equivalent to 0.1 milligram of iron.

Procedure.—1. Carefully pipet 0.5 c.c. of oxalated blood into a 50 c.c. volumetric flask.

2. Add 2 c.c. of the concentrated sulphuric acid. Mix.

3. Add 2 c.c. of the potassium persulphate solution. Mix.

4. Add approximately 25 c.c. of water. Mix.

5. Add 2 c.c. of the sodium tungstate solution. Mix.

6. Cool to room temperature and dilute to the graduation with water.

7. Mix thoroughly, then filter.

8. Pipet 20 c.c. of the clear filtrate into a 25 c.c. stoppered cylinder and label B.

9. Pipet 1 c.c. of the iron standard solution into another 25 c.c. stoppered cylinder and label S.

10. Add 0.8 c.c. of the concentrated sulphuric acid to the standard cylinder. Dilute with water to the 20 c.c. graduation. Cool to room temperature.

11. Add 1 c.c. of the potassium persulphate solution to each cylinder.

12. Add 4 c.c. of the potassium thiocyanate solution to each cylinder.

13. Mix and compare in the colorimeter.

Calculation:

$$\frac{S \times 50}{B} = \text{milligrams of iron per 100 c.c. of blood.}$$

or set B at 10 mm. when $S \times 5 =$ milligrams of iron per 100 c.c. of blood.

$$\frac{S \times 20}{B} = \text{volumes per cent of oxygen capacity of blood.}$$

Milligrams of iron per 100 c.c. of blood divided by 3.35 = grams of hemoglobin per 100 c.c. of blood.

Notes.—1. When the color of B in the iron determination is too light for comparison, the cup can be set at 25 millimeters when $S \times 2$ milligrams of iron per 100 c.c. of blood.

DETERMINATION OF BLOOD PHOSPHATASE
(Bodansky's Method)

Principle.—The difference between the amount of total inorganic phosphorus in the blood serum after its phosphoric esters have acted upon sodium glycerophosphate substrate and the amount determined without using a substrate is the quantity of phosphatase in the blood.

Materials:

Sodium beta-glycerophosphate, $Na_2C_3H_5(OH)_xPO_4:5\frac{1}{2}H_2O$.

Mono-sodium diethyl-barbiturate (veronal), $(C_2H_5)_2:C:(CONNa \cdot CONH):CO$.

Trichloreacetic acid, C.P., $CCl_3(COOH)$.

Molybdic acid (ammonia and phosphate free), MoO_3.

Stannous chloride, C.P., $SnCl_2$.

Mono-potassium phosphate, C.P., KH$_2$PO$_4$.

Acid Hydrochloric, HCl, Sp. Gr. 1.18.

Acid Sulphuric, H$_2$SO$_4$, Sp. Gr. 1.84.

Sodium Hydroxide, NaOH.

Solutions.—*Buffered Substrate Solution.*—Dissolve in a 500 c.c. volumetric flask 2.5 grams of sodium beta-glycerophosphate and 2.12 grams of monosodium-diethyl barbiturate in distilled water and dilute to the graduation. Mix thoroughly. Add 2-3 drops of chloroform and keep in a refrigerator.

10% Trichloracetic Acid Solution.—Dissolve 10 grams of trichloracetic acid in distilled water and dilute to 100 c.c.

10 Normal Sulphuric Acid Solution.—To approximately 500 c.c. of water in a beaker add 270 c.c. of sulphuric acid. When at room temperature dilute to a volume of 1 liter. This is approximately correct and sufficient for this purpose.

7.5% Sodium Molybdate Solution.—Place 90 grams of molybdic acid in a 2 liter volumetric flask. Add 500 c.c. of 10% sodium hydroxide solution (prepared as described in Chapter XXXIII). When the acid has dissolved dilute to the 2 liter graduation with water. Mix. The solution should be faintly alkaline to phenolphthalein. Allow to stand and decant the supernatant fluid for use.

Molybdic Acid Solution.—Transfer 3 c.c. of 10N sulphuric acid solution to a 25 c.c. glass stoppered cylinder. Add 3 c.c. of the 7.5% sodium molybdate solution. Mix. Add 6 c.c. of water. Mix. Prepare for each determination.

Stock Stannous Chloride Solution.—Transfer 30 grams of stannous chloride to a 50 c.c. of glass-stoppered volumetric flask. Add hydrochloric acid to make a solution and dilute to the graduation with the acid. This solution may be used for several months if kept in a refrigerator.

Dilute Stannous Chloride Solution.—Transfer 1 c.c. of stock stannous chloride solution to a 200 c.c. glass-stoppered volumetric flask. Dilute to the graduation with water. Mix. Prepare for each determination.

Stock Phosphate Solution.—Using an analytical balance weigh 0.439 gram of monopotassium phosphate. Transfer to a liter volumetric flask. Add 1 c.c. of concentrated sulphuric acid and dilute to the mark with water. Mix thoroughly. 10 c.c. contains 1 milligram of phosphorus.

Standard Phosphate Solution.—Transfer 10 c.c. of stock phosphate solution to a 250 c.c. volumetric flask. Dilute to the graduation with water. Add one drop of toluol. Mix. 5 c.c. contain 0.02 milligram of phosphorus.

Procedure.—1. Collect 10 c.c. sample of blood in a plain tube and allow to clot.

2. Centrifuge the blood and remove the serum.

3. Place 10 c.c. of the buffered substrate solution in a 25 c.c. test tube.

4. Place in a water bath or incubator at 37° C. for a few minutes.

5. Add 1 c.c. of blood serum, invert once, and replace in water bath for one hour.

6. Cool under running water.

7. Add 9 c.c. of 10% trichloracetic acid solution, mix, stand 10 minutes and

then filter. This is the total inorganic phosphorus filtrate. Label this filtrate TIP.

8. Pipet 2 c.c. of blood serum into another 25 c.c. test tube.

9. Add 9 c.c. of water and 9 c.c. of 10% trichloracetic acid solution. Mix. Allow to stand for 10 minutes, then filter. This is the serum inorganic phosphorus filtrate. Label SIP.

10. Pipet 5 c.c. of each filtrate into a respective test tube labeled SIP and TIP.

11. Into a similar test tube pipet 5 c.c. of standard phosphate solution.

12. To each of the 3 tubes add 4 c.c. of molybdic acid solution. Mix slightly.

13. To each tube add 1 c.c. of dilute stannous chloride solution. Invert each tube twice immediately. Allow to stand 3 minutes.

14. Compare in colorimeter.

Calculation.—Set TIP and SIP at 10 millimeters when if R equals the reading of the standard then

$$\text{Total inorganic phosphorus (TIP)} = R \times 0.8 = \text{milligrams phosphorus per 100 c.c.}$$

$$\text{Serum inorganic phosphorus (SIP)} = R \times 0.4 = \text{milligrams phosphorus per 100 c.c.}$$

Phosphatase units equal milligrams of inorganic phosphorus of the total inorganic phosphorus analysis (TIP) minus milligrams inorganic phosphorus of the serum inorganic phosphorus analysis (SIP).

One Bodansky unit equals the equivalent of one milligram inorganic phosphorus liberated from sodium glycerophosphate substrate during the first hour at pH 8.6 and at 37° C. per 100 c.c. of serum.

Notes.—1. The normal values for adults are in Bodansky units are 1.5 to 4 and for children 5 to 12.

2. Phosphatase units determined by different methods of analysis are not the same, therefore in reporting phosphatase units the name of the author of the method of analysis should always be mentioned.

3. Phosphatase units are increased in most conditions effecting the bones.

Polyostotic Paget's disease	50.0-135.0 units
Polyostotic Paget's disease with healing	15.0- 50.0 units
Localized Paget's disease	5.0- 20.0 units
Osteosclerosis fragilis	15.5- 21.3 units
(Marble bones, Abers-Schonberg disease)	
Infantile rickets	30.0-190.0 units
Healed rickets	6.0- 14.0 units
Multiple Myeloma	1.8- 28.1 units
Generalized osteoporosis (up to middle age).	5.0- 10.0 units
Senile osteoporosis	1.5- 4.0 units
Clinical hyperparathyroidism	about 25.0 units
Osteomalacia	High units
Hyperthyroidism with bone changes	High normal to slight increase

Erythroblastic anemia ....High normal to slight increase
Osteomyelitis ....Normal to slight increase
Fractures ....Normal to slight increase
Gaucher's disease ....Normal to slight increase
Arthritis ....High
Bone cysts ....High
Acromegaly ....High
Fragilitus osseum ....Normal
Non-bone cysts ....Normal
Achondroplasis ....Normal
Osteogenesis imperfecta ....Normal
Obstructive jaundice ....High
Other types of jaundice ....Normal

DETERMINATION OF CYSTINE IN FINGER NAILS

(*Sullivan*)

Principle: The color produced by treating the hydrolyzed nails with sodium naphthoquinon sulfonate is compared with a standard solution of cystine.

Apparatus.—Small acetylation flasks
      Oil bath
      Beakers, 50 c.c. capacity

Reagents.—1. 15N sulfuric acid, made by diluting about 430 c.c. of the concentrated acid to one liter.

2. 0.1N hydrochloric acid, made by diluting 10 c.c. of the concentrated acid to one liter.

3. Freshly prepared 5% solution of sodium cyanide.

4. Freshly prepared 0.5% solution of 1,2, naphthoquinon—4—sodium sulfonate.

5. 0.5N sodium hydroxide prepared by diluting 50 c.c. of 40% to one liter.

6. 10% sodium sulfite in 0.5N sodium hydroxide.

7. 2% sodium hyposulfite ($Na_2S_2O_4$) in 0.5N sodium hydroxide.

8. Carbex E.

Standard Solution.—Weigh exactly 200 mgm. of cystine and transfer to a one liter volumetric flask. Add about 500 c.c. of 0.1N hydrochloric acid. Shake until dissolved and dilute to the mark with 0.1N hydrochloric acid. This solution keeps well in the ice box, and contains one mgm. of cystine per 5 c.c.

Procedure.—1. Clean the finger nail clippings by gently rubbing with the blunt edge of a scalpel or similar instrument, then immerse for a short time in acetone, decant the acetone, dry by fanning, and cover with ether. Stir for about 5 minutes, decant the ether and dry the clippings.

2. Cut the clippings into small bits and weigh 50 mgm. on an accurate analytical balance.

3. Transfer the weighed, finely cut clippings to a small flask, add 0.15 c.c. of 15N sulfuric acid and digest on an oil bath for one hour at 150° C.

4. Wash the dark hydrolisate into a small beaker with 5 c.c. of water, add about 25 mgm. of carbex E, heat to a gentle boil and filter, catching the filtrate in a 25 c.c. graduated cylinder. The filtrate should be clear and colorless.

5. Wash the filter with 3 c.c. of 0.1N hydrochloric acid, followed by water until the filtrate reaches the 25 c.c. mark.

6. Mix the filtrate which is now ready for color development.

7. In a test tube marked S pipette 5 c.c. of the cystine standard.

8. In a test tube marked B pipette 5 c.c. of the clarified filtrate.

9. To each add 2 c.c. of 5% sodium cyanide, mix and wait ten minutes. Then add 1 c.c. of 0.5% 1,2 naphthoquinone—4—sodium sulfonate, mix, add 5 c.c. of 10% sodium sulfite in 0.5N sodium hydroxide; mix and let stand 30 minutes. Now add 1 c.c. of a 2% sodium hyposulfite solution in 0.5N sodium hydroxide whereupon the reddish brown color becomes a purer red.

10. Compare in colorimeter setting standard at 20.

Calculation:

$$\frac{20 \times 1 \times 5 \times 100}{R \times W} = \frac{10,000}{R \times W} = \text{per cent cystine in nails.}$$

Where R = reading of the unknown
W = weight of sample taken in milligrams

Notes:

1. The 1.2 naphthoquinone—4—sulfonate must be quite pure. It should give only a pale yellow color when 2 c.c. of a 1% aqueous solution are treated with the reagents in the absence of cystine.

2. The cyanide should be at least 95% and practically free from iron.

3. The sodium hyposulfite must have good reducing power. It tends to decompose and its reducing power should be checked from time to time against indigo.

4. Cleaning mixtures should not be used on the glassware. For cleaning purposes use only hot distilled water.

5. The presence of reducing material such as hydrogen sulfide is to be avoided since it interferes with the progress of the color reaction.

6. The normal cystine content of finger nails ranges from 10 to 13%. It is frequently low in chronic arthritis.

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CHAPTER XXXV

METHOD FOR THE DETERMINATION OF BASAL METABOLIC RATE

Principle.—The rate of absorption of oxygen by the blood from the lungs under specified conditions is determined by either the open or the closed method.

1. *Open Method.*—The patient inspires ordinary outdoor air and all air that he expires during a known period of time is caught in a suitable receptacle and measured. A sample is removed and analyzed for oxygen, carbon dioxide, and nitrogen in the Haldane-Henderson gas analysis apparatus, and the oxygen absorption and carbon dioxide excretion of the patient is calculated. For this method, which requires training in gas analysis, see Peters and Van Slyke,[1] Volume II, Chapter 5.

2. *Closed Method.*—The patient breathes into and out of a container of oxygen in which the carbon dioxide he produces is absorbed, either for a fixed length of time, during which the decrease in volume of oxygen is noted (Benedict-Roth and Sanborn apparatuses), or during the absorption of a fixed volume, one liter, of oxygen, for which the time required is noted (Jones apparatus). From the rate of absorption of oxygen, the rate of heat production per hour is determined, and compared with that of a normal individual of the same biometric measurements. The percentage variation from the latter is called the basal metabolic rate.

Materials.—*Apparatus.*—Closed circuit respiration apparatus, with mouthpiece (sterilized by boiling), nose clip, thermometer, kymograph with recording paper and ink. Bed, couch, stretcher, or other object upon which patient may recline comfortably. Watch. Clinical thermometer. Barometer calibrated in millimeters. Scales for weighing patient. Measuring device for obtaining his height.

Chemicals.—Soda lime. Oxygen, pure, in compression cylinder.

Procedure.—If the patient is in the hospital he is given a light supper the night before the determination: is given no breakfast: is kept in bed: is taken to the metabolism station on a stretcher, and is given absolute rest for a half hour before the determination. An out-patient is told to eat a light supper at least 12 or 14 hours before the test; to eat nothing after this meal; to get a good night of rest; to eat no breakfast; and to dress and come to the laboratory in a leisurely manner, riding if possible. He must not exert himself. Upon arrival he is given absolute rest for 30 or 40 minutes before the test.

Wrap recording paper tightly around kymograph drum and paste in place either with a gummed strip or with paper stickers. Fill recording pen with ink.

By means of rubber tube, connect oxygen compression cylinder to the petcock

[1] *Quantitative Clinical Chemistry.* Williams and Wilkins, 1932.

on the apparatus for introducing oxygen gas. Admit oxygen into apparatus until pen is within a few centimeters of the edge of the paper drum. Shut valve on oxygen tank and petcock on apparatus. Connect patient with mouthpiece of apparatus and start motor. Adjust nose clip. Start kymograph revolving. Record temperature of gas in apparatus, and pulse of patient. At the end of 8 minutes remove nose clip and mouthpiece from patient. Stop motor and kymograph. Record temperature of gas in apparatus. Record barometric pressure. Determine patient's nude weight and his temperature. Record sex of patient. Record his age in years to nearest birthday.

Calculation.—Companies manufacturing apparatus supply direction booklets and tables for making calculations. In case a check on calculations is desired, the apparent volume of oxygen used by the patient can be converted to the true volume of dry gas at 0° C. and 760 mm. pressure by multiplying by the factor F,[2] where $F = 0.0012 \times B + 0.001 \; [4.6(30+t°) - 52]$, B is the observed barometric pressure in millimeters, and t° is the observed temperature of the gas in degrees Centigrade.

Each liter of oxygen under standard conditions represents 4.825 calories of heat production (the respiratory quotient being assumed equal to 0.82). Therefore the heat production of the patient per hour is

$$C = \frac{F \times V \times 0.004825 \times 60}{M} \text{ Calories,}$$

where F has the value found above, and V is the apparent number of c.c. of oxygen absorbed by the patient in M minutes.

The number of calories of heat which should be produced by an individual of the same biometric measurements can be found from the manufacturers' tables mentioned above, or in Peters and Van Slyke, Volume II, pp. 207-214. If the normal number of calories is N, the patient's basal metabolic rate is

$$\frac{C - N}{N} \times 100 \text{ percent.}$$

Notes.—1. It is advisable in case the patient has never had a basal metabolic rate determination to make a practice run to accustom him to the procedure. This run may be made in the afternoon. Its results are of course discarded.

2. For accurate results the patient must be completely relaxed, and must make no movements during the determination.

3. The patient's temperature should be noted, as the metabolic rate rises about 6% with each degree Fahrenheit of fever.

4. A basal metabolic rate between − 10 and + 15 cannot be considered definitely pathological.

5. The determination of basal metabolic rate is of clinical value principally in diagnosing and following the effect of treatment (surgical, radiological, or medical) of disorders of the thyroid gland. In hyperthyroidism the rate is above normal; in hypothyroidism the rate is below normal. Determinations may also be of value in disorders of some of the other glands of internal secretion.

[2] Or see Peters and Van Slyke, Vol. II, p. 129.

CHAPTER XXXVI

METHODS FOR THE CHEMICAL EXAMINATION OF MILK AND OTHER FOODS

Ordinarily milk examinations are made to determine the nutritive value and this information is obtained by estimating the specific gravity. total solids. ash, fat, protein, and lactose.

When foreign ingredients or adulterants are present in milk, special methods are employed to detect them.

COLLECTION AND SAMPLING OF COW'S MILK

1. Great care is necessary to secure a homogeneous sample. In the case of bottled milk, collect one or more bottles as prepared for sale. In sampling bulk milk. thoroughly mix by pouring from one clean vessel into another three or four times. If this procedure is impracticable, thoroughly stir the milk for at least one-half minute with a suitable appliance long enough to reach to the bottom of the container. If cream has formed on the milk. continue the mixing until all cream is detached from the sides of the vessel and evenly emulsified throughout the liquid.

2. Place the samples in nonabsorbent. air-tight containers and keep them in the cold, but at a temperature above freezing, until ready for examination. When transported by mail, express, or otherwise, the containers should be completely filled, tightly stoppered, and marked for identification. A necessary quantity of preservative (corrosive sublimate, potassium dichromate, or formaldehyde) may be used, except where the presence of the preservative may be objectionable in connection with physical or chemical tests to be applied in addition to the determination of fat.

3. The quantity of sample required will depend upon the number of determinations to be made. For the usual analysis collect 250 to 500 c.c. (½ to 1 pint) of sample; for the fat determination only, 50 to 60 c.c. (approximately 2 fluid ounces) will suffice.

4. Before withdrawing portions for analytical determinations, bring the sample to a temperature of 15° to 20° C. and mix thoroughly by pouring into a clean receptacle and back until a homogeneous mixture is assured. If lumps of cream do not completely disappear, warm the sample to about 38° C., mix thoroughly, then cool to 15° to 20° C. In case a measured volume is required in a determination, bring the temperature of the sample to 20° C. before pipeting.

DETERMINATION OF SPECIFIC GRAVITY

This is most readily obtained with the aid of a hydrometer, accurately graduated within the limits of the widest possible variation in the specific gravity of milk.

Hydrometers for special use with milk are known as lactometers. They usually have a thermometer combined with them and the readings should be made at the temperature specified on the lactometer (usually 60° F., 15.6° C.) by immersing the cylinder containing the milk in tap water.

DETERMINATION OF TOTAL SOLIDS

A platinum dish is desirable, if not available porcelain may be used.

1. Weigh dish on the chemical balance.

2. Add about 5 c.c. of the thoroughly mixed sample of milk and weigh again to milligrams. This weighing should be done as rapidly as possible to avoid inaccuracies due to evaporation.

3. Place dish on a hot water bath until dry (about 2 hours) then for about ten minutes in a hot air oven (100°-105° C.).

4. Cool to room temperature in a desiccator and weigh.

5. Multiply the weight of the residue by 100 and divide by the weight of the sample taken to obtain the per cent of total solids in the milk.

Note.—Prolonged heating in the drying oven should be avoided as this tends to decomposition. This is indicated by the formation of a brownish color. The residue should be nearly pure white.

DETERMINATION OF ASH

1. The dish containing the milk residue (above) is placed on a triangle supported on a ring stand and ignited at a dull red heat by means of a Bunsen burner until a perfectly white ash is obtained.

2. Cool in a desiccator to room temperature and weigh.

3. Multiply the weight of the residue by 100 and divide by the weight of the original sample to obtain the per cent of ash in the milk.

BABCOCK METHOD FOR ESTIMATING THE FAT OF COW'S MILK

Principle.—Strong sulphuric acid is added to the milk to dissolve the serum solids and set free the fat from its emulsion. The fat is then permitted to rise into the graduated neck of a Babcock bottle and the percentage read directly.

Reagent and Apparatus.—1. Commercial concentrated sulphuric acid with a specific gravity of 1.82 to 1.83 at 20° C.

2. Special Babcock milk pipet (Fig. 362) graduated to deliver 17.5 c.c. but which holds 17.6 c.c. to the graduation mark, the extra 0.1 c.c. being allowed for the milk which clings to the walls.

3. Standard Babcock test bottle (Fig. 363).

4. Acid measure (Fig. 364) graduated to hold 17.5 c.c.

5. Centrifuge for the Babcock bottles. Special trunnion cups may be purchased for use with the International Centrifuge.

Procedure.—1. Transfer 17.6 c.c. (equivalent to 18 grams) of well-mixed milk to a milk test bottle by means of the special pipet. The milk remaining in the pipet tip is blown out.

2. Add 17.5 c.c. of sulphuric acid, preferably not all at one time, pouring it down the side of the neck of the bottle in such a way as to wash any traces of the milk into the bulb. The temperature of the acid should be about 15° to 20° C.

3. Mix by rotation until all traces of curd have disappeared; then transfer the

FIG. 362.—SPECIAL BABCOCK MILK PIPET

FIG. 363.—BABCOCK MILK TEST BOTTLE
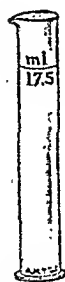
FIG. 364.—ACID MEASURE FOR THE BABCOCK METHOD
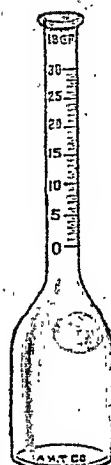
FIG. 365.—CREAM TEST BOTTLE

bottle to the centrifuge; counterbalance it; and, after the proper speed has been attained, whirl five minutes.

4. Add soft water at 60° C., or above, until the bulb of the bottle is filled.

5. Whirl two minutes.

6. Add hot water until the liquid column approaches the top graduation of the scale.

7. Whirl one minute longer at a temperature of 55° to 60° C. Transfer the bottle to the warm water bath maintained at a temperature of 55° to 60° C., immerse it to the level of the top of the fat column, and leave it there until the column is in equilibrium and the lower fat surface has assumed a final form.

8. Remove the bottle from the bath; wipe it; and measure the fat column, in terms of percentage by weight, from its lower surface to the highest point of the upper miniscus.

9. The fat column, at the time of measurement, should be translucent, of a golden yellow or amber color, and free from visible suspended particles. R

tests in which the fat column contains milk or shows the presence of curd or of charred matter, or in which the reading is indistinct or uncertain.

Estimation of Fat in Cream.—1. Because of variation in specific gravity of cream and its high viscosity, 18-gram samples can be accurately taken only by weighing, but with cream containing between 20 and 30% fat the sample is sufficiently accurate for routine work by measuring 18 c.c. of cream with a special pipet. Use the special cream test bottle shown in Figure 365.

2. Proceed as with milk except that readings are made at the bottom of the upper meniscus. Liquid petrolatum added will flatten out the meniscus and make the reading easier.

Notes.—1. In adding the acid, the test bottle is conveniently held at an angle so that the acid will run down the wall of the bottle and not in a small stream into the center of the milk, the bottle being slowly turned around and the neck thus cleared of adhering milk. The milk and the acid in the test bottle should be in two distinct layers without much of a black band of partially mixed liquids between them. Such a dark layer is often followed by an indistinct separation of the fat in the final reading. The cause of this may be that a partial mixture of acid and milk before the acid is diluted with the water of the milk, will bring about too strong an action of the acid in this small portion of the milk, and thus char the fat contained therein. The appearance of black flocculent matter in or below the column of fat renders a correct measurement difficult and at times even impossible; if the black specks occur in the fat column itself, the readings are apt to be too high; if below it, the difficulty comes in deciding where the column of fat begins.

2. The acid should be carefully mixed with the milk by giving the test bottle a rotary motion. In doing this care should be taken that the liquid is not shaken into the neck of the test bottle. When once begun the mixing should be continued until completed; a partial and interrupted mixing of the liquids will often cause more or less black material to separate with the fat when the test is finished. Clots of curd which separate at first by the action of the acid on the milk must be entirely dissolved by continued and careful shaking.

ESTIMATION OF TOTAL NITROGEN OF COW'S MILK

Principle.—Organic compounds are oxidized and the nitrogen converted into ammonia which is distilled off into a standard acid solution and titrated with standard alkali solution.

Procedure.—1. Place 5 grams of the milk, weighed accurately, in a 500 c.c. Kjeldahl digestion flask.

2. Add approximately 0.7 gram of mercuric oxide, or 0.1 gram of crystallized copper sulphate, 10 grams of potassium sulphate (or 10 grams of anhydrous sodium sulphate) and 25 to 30 c.c. of sulphuric acid, specific gravity 1.84.

3. Place the flask in an inclined position and heat below the boiling point of the acid until frothing has ceased. (A small piece of paraffin may be added to prevent extreme frothing.) Then increase the heat until the acid boils briskly and

digest for a time after the mixture is colorless or nearly so. or until oxidation is complete (Fig. 366).

4. Cool. dilute with about 200 c.c. of water, cool again. add a few pieces of granulated zinc or pumice stone. if necessary to prevent bumping. and 25 c.c. of a 4% solution of potassium sulphide with shaking. (When no mercury or mercuric oxide is used the addition of the potassium sulphide solution is unnecessary.)

5. Next add sufficient saturated solution of sodium hydroxide free from nitrate,
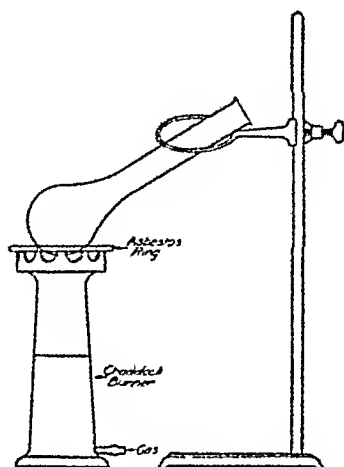
FIG. 366.—SUPPORT FOR KJELDAHL DIGESTION
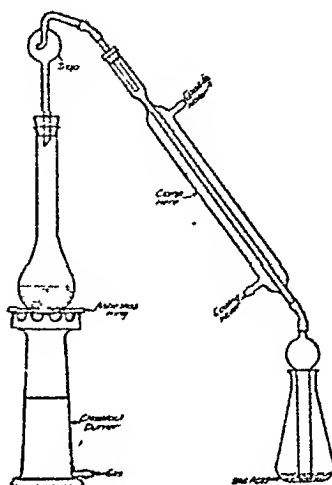
FIG. 367.—APPARATUS FOR THE DISTILLATION OF AMMONIA FOR NITROGEN DETERMINATION

o make the reaction strongly alkaline (50 c.c. are usually enough), pouring it own the side of the flask so that it does not mix at once with the acid solution.

6. Connect the flask by means of a Kjeldahl connecting bulb with a condenser Fig. 367), mix the contents of the flask by shaking, and distill until all ammonia as passed over into 50 c.c. of standard N/10 hydrochloric or sulphuric acid. he first 150 c.c. of the distillate will generally contain all the ammonia.

7. Titrate with N/10 standard alkali solution, using methyl red or cochineal olution as indicator.

8. Multiply the percentage of nitrogen by 6.38 to obtain the equivalent percentage to be reported as milk proteins.

Calculation:

$$\frac{50 - \text{c.c. N/10 sodium hydroxide} - \text{c.c. blank} \times 0.14}{\text{weight of sample}} = \text{per cent total nitrogen}$$

Notes.—1. Determinations are to be made in duplicate and blanks are to be run. ising about 1 gram of cane sugar instead of the unknown. Sugar aids in the reduction of any nitrates that may be present in the reagents.

2. The flame of the burner should strike only the portion of the flask below the evel of the acid. Sheet iron or asbestos board with a hole in it serves well as a upport.

3. The Kjeldahl flask should be fitted with a rubber stopper through which passes the lower end of a Kjeldahl connecting trap bulb to prevent sodium hydroxide being carried over mechanically during the distillation. The bulb should be about 3 centimeters in diameter and the tubes should be of the same diameter as the condenser tube with which the upper end of the bulb is connected by means of rubber tubing. A piece of glass tubing about 12 centimeters long of the same diameter as the condenser tubing is attached to the lower end of the condenser by means of rubber tubing and should reach nearly to the bottom of the Erlenmeyer flask. This delivery tube is capable of being detached from the condenser for purposes of rinsing. It is preferable that the distilling system be made of Pyrex glass.

DETERMINATION OF CASEIN OF COW'S MILK

1. This determination should be made while the milk is fresh, or nearly so. When it is not practicable to make this determination within 24 hours, add 1 part of formaldehyde to 2500 parts of milk and keep in a cool place.

2. Place 10 grams of the sample in a beaker with 90 c.c. of water at 40° to 42° C. and add at once 1.5 c.c. of dilute acetic acid $(1+9)$. Stir, and let stand 3 to 5 minutes.

3. Decant on a filter, wash by decantation two or three times with cold water, and transfer the precipitate to the filter. Wash once or twice on the filter.

4. The filtrate should be clear, or very nearly so. If the first portions of the filtrate are not clear, repeat the filtration, after which complete the washing of the precipitate.

5. Transfer without loss to a Kjeldahl flask and determine nitrogen in the washed precipitate and filter paper as described above for total nitrogen. Multiply by 6.38 to obtain the equivalent of casein.

6. To a sample of milk that has been preserved, the acetic acid should be added in small portions, a few drops at a time, with stirring, and the addition should be continued until the liquid above the precipitate becomes clear, or very nearly so.

ESTIMATION OF MILK SUGAR

Principle.—The reducing action of the clarified milk is measured by a definite amount of Fehling's solution.

Reagents.—A. *Fehling's Copper Solution*—34.639 gms. C. P. copper sulfate are dissolved in water in a 500 c.c. volumetric flask and the solution is then diluted to the mark.

B. *Fehling's Alkaline Tartrate Solution*—173 gms. Rochelle salts and 50 gms. sodium hydroxide are dissolved in water and diluted to 500 c.c.

Procedure.—1. Place 25 gms. milk in a 250 c.c. flask, add 0.5 c.c. 30% acetic acid and shake well. After standing a few minutes about 100 c.c. of boiling water are run in, the flask shaken and 25 c.c. of alumina cream added. Let stand about 10 minutes. Pour through a ribbed filter paper, catching filtrate in a 250 c.c. volumetric flask. Wash the precipitate thoroughly allowing the washings to run

into the volumetric flask which is then filled to the mark. The filtrate should be perfectly clear.

2. Fill a 50 c.c. buret to the mark with this filtrate.

3. In a 250 c.c. porcelain casserole place exactly 5 c.c. each of solutions A and B. Add about 40 c.c. of water and heat to boiling.

4. While still boiling add the milk filtrate from the burêt, a small portion at a time until all the copper is reduced. This is indicated by a gradual color change from deep blue through green to dull red. The first appearance of this color is the end point.

5. Note from buret the volume of milk filtrate used.

Calculation.—Ten c.c. of the above Fehling's mixture is equal to 0.067 gm. of milk sugar. The milk filtrate represents a tenfold dilution of the milk so that 0.067 multiplied by 10 and divided by the volume of filtrate used represents the percent of lactose in the milk.

Notes.—1. Practice will soon enable the eye to judge the end point quite definitely.

2. The sugar-containing solution may be added quite rapidly until the solution becomes pale green, when the solution should be added cautiously, a few drops at a time.

DETECTION OF PRESERVATIVES IN COW'S MILK

Phenylhydrazin Test for Formaldehyde.—1. To a portion of the sample add an equal volume of strong alcohol, shake and filter from any insoluble matter.

2. To 5 c.c. of the filtrate add 0.03 gram of phenylhydrazin hydrochloride and 4 or 5 drops of a 1% ferric chloride solution.

3. Mix, add slowly with agitation, in a bath of cold water to prevent heating the liquid, 1 to 2 c.c. of concentrated sulphuric acid.

4. Dissolve the precipitate by the addition of either concentrated sulphuric acid (keeping the mixture cool) or alcohol. In presence of formaldehyde a red color develops.

Ferric Chloride Test for Salicylic Acid.—1. Acidify 100 c.c. of the milk with 5 c.c. of hydrochloric acid (1 + 3), shake until curdled, filter, and extract with 50 to 100 c.c. of ether.

2. Wash the ether layer with two 5 c.c. portions of water, evaporate the greater portion of the ether in a porcelain dish on a steam bath, allow the remainder to evaporate spontaneously, and add a drop of 0.5% ferric chloride solution.

3. A violet color indicates salicylic acid.

Test for Benzoic Acid.—1. Acidify, filter, and extract a 100 c.c. portion of the milk with ether as directed for salicylic acid. If benzoic acid is present in considerable quantity, it will crystallize from the ether in shining leaflets having a characteristic odor on heating.

2. Dissolve the residue in hot water, divide into two portions, and test as directed below. The residue may also be purified by sublimation and the melting point determined.

(a) Make the solution alkaline with ammonium hydroxide, expel the excess of ammonia by evaporation, dissolve the residue in water, and add a few drops of a neutral 0.5% ferric chloride solution. A brownish precipitate of ferric benzoate indicates the presence ol benzoic acid.

(b) Add to the water portion 1 or 2 drops of a 10 per cent solution of sodium hydroxide and evaporate to dryness. To the residue add 5 to 10 drops of concentrated sulphuric acid and a small crystal of potassium nitrate. Heat for ten minutes in a glycerol bath at 120° to 130° C., or for 20 minutes in a boiling water bath. The temperature must not exceed 130° C. After cooling add 1 c.c. of water and make distinctly ammoniacal; boil the solution to decompose any ammonium nitrate that may have been formed. Cool and add a drop of fresh, colorless ammonium sulphide, without allowing the layers to mix. A red-brown ring indicates benzoic acid. On mixing, the color diffuses through the whole liquid and, on heating, finally changes to greenish-yellow. This differentiates benzoic acid from salicylic acid or cinnamic acid. The last two form colored compounds, which are not destroyed by heating.

Test for Borax and Boric Acid.—1. Immerse a strip of turmeric paper in the sample acidified with hydrochloric acid in the proportion of 7 c.c. of strong acid to reach 100 c.c. of sample and allow the paper to dry spontaneously.

2. If borax or boric acid is present, the paper will acquire a characteristic red color, changed by ammonium hydroxide to a dark blue-green, but restored by acid.

COLLECTION AND CHEMICAL ANALYSIS OF HUMAN MILK

Collection.—There are two methods of obtaining samples of breast milk for analysis:

1. Express all the milk from one breast and mix thoroughly.

2. Draw 1 ounce of milk before nursing and 1 ounce after nursing. Mix the two samples thoroughly. The best time for obtaining samples is 9 to 10 A.M.

Determination of Specific Gravity.—Determined most conveniently by means of a Soxhlet, Veith or Quevenne lactometer at 60° F. The lactometer reading is corrected by adding 0.0001 for every degree F. above 60° and subtracting 0.0001 for every degree F. below this temperature.

Determination of Percentage of Fat.—This is essentially a modification of the Babcock test previously described except that a smaller bottle is employed (Fig. 368); the technic is practically the same. Otherwise the regular Babcock bottle and method are to be preferred if sufficient sample is available.

1. By means of a special narrow pipet, introduce milk up to the 5 c.c. mark.

2. Add sufficient commercial sulphuric acid (specific gravity 1.83) to fill the body of the tube and rotate to secure a homogeneous mixture.

3. Fill the neck of the tube with a mixture consisting of equal volumes of amyl alcohol and concentrated hydrochloric acid and centrifuge for 2 minutes, etc., as described above.

4. The fat collects in a column in the upper part of the acid alcohol mixture and the percentage is read off directly on the graduated stem.

Determination of Total Solids.—1. Introduce 2 to 5 grams of milk into a weighed flat-bottomed platinum dish and quickly ascertain the weight to milligrams.

2. Carefully expel most of the water by heating in a water bath and heat for about 5 min. in an oven regulated to a temperature between 100° and 105° C. Cool and weigh.

3. Divide the weight of the residue in grams by the weight of the milk used. Multiply the result by 100 to give the per cent of total solids in the milk.

Determination of Ash.—1. Heat the dry solids obtained above over a low flame (care should be taken that the dish is not heated above dull redness) until a white or light gray ash is obtained.

2. Cool the dish in a desiccator and weigh.

3. Divide the weight of the ash by the weight of the original milk taken for total solids and multiply by 100 to obtain the per cent of ash in the milk.

Determination of Total Nitrogen (Proteins).—1. Introduce 5 grams of milk into a 500 c.c. Kjeldahl flask and carry out the nitrogen determination as described for cow's milk.

2. Multiply the per cent of nitrogen by the factor 6.37 to obtain the protein content of the milk.

Determination of Lactose.—1. Introduce 1 c.c. of milk into a 100 c.c. volumetric flask.

2. Add 2 c.c. of 10% sodium tungstate.

3. Add gradually 2 c.c. of two-thirds normal sulphuric acid or 16 c.c. of N/12 acid, mix well and let stand five minutes.

4. Dilute to the mark, mix and filter.

Fig. 368.—Babcock Fat Bottle for Human Milk

5. Into a Folin-Wu sugar tube introduce 1 c.c. of the filtrate and add 1 c.c. of water. Into another tube place 2 c.c. of standard lactose solution. Add 2 c.c. of the Folin-Wu alkaline copper reagent (see page 729) to each tube and heat in boiling water for 8 minutes. Cool and add 2 c.c. of phosphomolybdic reagent (see page 729) to each tube. Dilute to 25 c.c. mark and compare in colorimeter.

6. Calculate as follows:

$$\frac{20}{R} \times 0.6 \times \frac{100}{0.01} \times \frac{1}{1000} = \text{per cent lactose} = \frac{120}{R} = \text{per cent lactose}$$

7. For the standard lactose solution, prepare a stock lactose solution by dissolving 1 gram of lactose (weighed on an analytical balance) in 0.2% benzoic acid and making up to 100 c.c. in a volumetric flask. The working standard is prepared by diluting 3 c.c. of this stock solution in a 100 c.c. volumetric flask to the mark with 0.2% benzoic acid. Two c.c. of this solution equal 0.6 milligram lactose.

CHEMICAL ANALYSES OF DIABETIC FOODS

Preparation of Sample.—Grind sufficiently fine in a mortar or mill to pass through a 1 millimeter sieve. Preserve in a sealed container to prevent moisture changes.

Detection of Starch.—Starch is best detected, when present to any appreciable extent in any mixture, by boiling a portion of the sample in water, cooling and applying a solution of iodine. A characteristic blue color appears if starch is present. Very small amounts of starch are best identified by adding iodine solution to powder on a microscopic slide, or better, to the power previously rubbed with water on a slide under a cover glass; the starch granules, if present, will be colored intensely blue by the iodine, and are at once rendered apparent when viewed through the microscope.

Estimation of Moisture.—Weigh accurately 2 to 3 grams of foodstuff on a tared watch glass and dry to constant weight in an oven at 105° to 106° C. This requires about four hours. The loss in weight represents moisture.

Estimation of Ash.—Follow the procedure for determining the ash of human milk.

Estimation of Protein.—Determine the total nitrogen of a 1 gram sample according to the procedure given for the total nitrogen of milk. Calculate the protein by multiplying the total nitrogen by the appropriate factor which varies with different cereals as follows: wheat, 5.70; rye, 5.62; oats, 6.31; corn, 6.39; and barley, 5.82. If the sample represents a mixture of various grains the conventional factor 6.25 is employed.

Estimation of Fat.—Heat on a boiling water bath for ninety minutes 5 grams of the sample in a 200 c.c. flask with 50 c.c. of water and 2 c.c. of 25% hydrochloric acid (specific gravity 1.125). Cool, nearly neutralize with 40% sodium hydroxide (using 0.04% methyl orange as indicator) and filter. Transfer contents to a filter paper and wash with hot water. Dry filter and contents in oven at 105° C. and transfer to extraction thimble of Soxhlet extractor. Extract with ether for 16 hours, transfer the extract to a weighed beaker, evaporate the ether on the water bath and dry to constant weight in the oven. This increase in weight represents the fat.

Estimation of Starch, Sugar and Dextrin.—1. Extract 4 to 5 grams of the fine powder (accurately weighed) on a hardened filter paper with five successive 10 c.c. portions of ether.

2. Wash the powder into a beaker with 50 c.c. of water.

3. Immerse the beaker into a boiling water bath for 15 minutes or until all the starch is gelatinized.

4. Cool to 55° C.

5. Add 20 c.c. of a fresh 0.5% aqueous solution of U.S.P. pancreatin.

6. Digest at 55° C. for one hour.

7. Heat again to boiling for a few minutes to gelatinize the remaining starch granules.

8. Cool to 55° C. and redigest at this temperature with another 20 c.c. portion of pancreatin solution for one hour or until the residue treated with iodine gives no test for starch.

9. Cool. make up to 250° C. and filter.

10. Place 200 c.c. of the filtrate into a flask, add 20 c.c. of dilute hydrochloric acid (specific gravity 1.125; made by adding 2 volumes of water to 5 volumes of concentrated acid) connect with a reflux condenser and heat in a boiling water bath for two and a half hours.

11. Cool, nearly neutralize with 10% sodium hydroxide, finish the neutralization with sodium carbonate and dilute to 500 c.c.

12. Mix the solution, pour through a dry filter and determine the dextrose in an aliquot part according to Benedict's method for sugar in urine.

Calculation: The amount of dextrose found multiplied by 0.9 gives the amount of starch. This amount multiplied by the aliquot part taken and the two dilutions gives the starch in the original sample from which the percentage may then be calculated.

CHAPTER XXXVII

METHODS FOR TOXICOLOGICAL EXAMINATIONS

Principles.—1. It is highly important that a sufficient amount of material be furnished. Depending on the expected concentration of the substance, 500 to 2000 c.c. of urine or 100 to 200 grams of feces are desired. In cases of acute mercury poisoning with oliguria, small amounts of urine can be examined when more is not obtainable.

2. Material desirable for detection of some common poisons are as follows:

(a) *Lead:* Feces and urine. Feces contain more than urine.

(b) *Mercury:* Urine, feces and stomach contents. Excretion about equal in urine and feces. Urine is desired when obtainable, as analysis is more quickly and easily done.

(c) *Arsenic:* Urine, some in feces.

(d) *Morphine:* Feces and urine.

(e) *Methyl alcohol:* Urine.

(f) *Carbon monoxide:* Blood.

(g) *Alkaloids, phenol, iodine, alkalies, etc.:* Vomitus and stomach washings.

(h) *Autopsy material:* Part of liver, brain, one kidney, stomach and intestinal contents.

REINSCH METHOD FOR THE DETECTION OF ARSENIO, ANTIMONY AND MERCURY

The Reinsch test possesses two advantages: (1) as a preliminary test when abundance of material is available and (2) for clinical purposes during the life of the patient. It is a test not only for arsenic, but for mercury and antimony as well. It may be applied directly to a liquid containing organic matter, as the urine, and may be completed in a few minutes. The Reinsch test can also be applied to tissue.

Principle.—If a solution containing an arsenite and acidulated with about one-fifth its volume of hydrochloric acid (arsenic free) is heated a little below the temperature of boiling water for one half hour in the presence of metallic arsenic free copper foil (*keep copper below surface of liquid*), a gray stain is formed upon the copper which is an alloy of copper and arsenic. It is not formed in the presence of powerful oxidizing agents such as the chlorates. With an arsenate it is only slowly formed. If the presence of arsenates be suspected, it is well to reduce them to arsenites by sulphur dioxide and expel the excess of gas by boiling before applying the test. A stain having an appearance similar to that caused by arsenic

is also formed if the liquid contains compounds of sulphur, selenium, gold, platinum, silver, bismuth, antimony or mercury.

To distinguish the arsenical stain from the others, the strip of thin copper foil, which should be about one-eighth by three-quarters of an inch, is taken from the solution, gently washed and dried by contact with filter paper. It is then inserted into one end of a clean piece of thin glass tubing, open at both ends and about 8 inches long. This is held at an angle of about 10 degrees to the horizontal and gently warmed along its entire length until the interior of the tube and the foil are perfectly dry. The portion of the tube immediately above the copper is then slightly warmed (to insure the formation of larger crystals than would be deposited upon a cold surface) and then, the forefinger being more or less applied to the upper opening in such a manner as to allow a very slow current of air to go through the tube, the copper is heated strongly. There is danger of loss if the air current is too rapid.

Of the compounds mentioned, selenium, arsenic, antimony and mercury are the only ones which produce a sublimate in the tube. Sulphur is volatilized as sulphur dioxide, and the other metals remain upon the copper.

The sublimate produced by mercury is grayish rather than pure white, and, when examined with the microscope the mercury deposit when rubbed, assumes a silvery appearance. Arsenic, antimony and bismuth give a gray or black deposit, is found to consist of an aggregation of shining globules. The deposits of the oxides of arsenic, antimony and selenium are white and more closely resemble each other, but differ in certain particulars. The *antimonial deposit* is nearer to the point at which heat was applied than the arsenical, and a portion of it may be in that part of the tube which was in the flame. After the formation of the sublimate it may be readily driven along the tube by a moderate heating if it be *arsenic*, while much higher temperature is required to volatilize the antimonial deposit. The arsenical deposit consists entirely of brilliant octahedral crystals varying in size, the larger being in the portion of the sublimate nearest to where heat was applied. The crystals are bright, with finely defined edges, and scintillate when the tube is rotated on its axis in the sunlight. The *antimonial deposit* is generally entirely amorphous. It may, however, contain crystals some of which may be octahedral of the same shape as the arsenical crystals, but rather duller in luster and less transparent, and whose edges appear as broader black lines. These crystals, if present at all, are always few in number and are surrounded by much granular material, and require a high temperature for their volatilization. Occasionally prismatic crystals are also formed either beyond the copper or in that part of the tube which was in the flame.

The presence of *selenium* is exceptional, originating most frequently as an impurity of sulphuric acid. Microscopically its sublimate is found to consist of amorphous material and may contain prismatic crystals arranged in feathery bundles.

Two points are to be borne in mind: Hydrochloric acid is rarely free from arsenic and the copper foil may contain it. The method should, therefore, never be

used without a blank. If the chemicals be pure the copper is, if anything, brightened. Should it become dimmed in the slightest degree, the acid, which is usually at fault, must be rejected. An objection to this method is the fact that copper is introduced into the articles under examination. It should therefore never be used except with a small sample of the available material.

The practical limit of delicacy of this test is about 0.0065 milligram. It is certainly inferior in delicacy to the Marsh test.

It should also be remembered that normal urine may and frequently does, contain arsenic. The amount of this so-called normal arsenic is variable, depending upon food, occupation and environment. The Reinsch test is hardly sufficiently delicate to detect this "normal" arsenic, but the Marsh test usually will give a test for arsenic with normal urine.

In the case of tissue, 5 to 10 grams of finely divided material are diluted with distilled water. The mixture is then acidulated with concentrated hydrochloric acid and the Reinsch test conducted in the same manner as described.

METHOD FOR THE DETECTION OF LEAD

Preliminary Treatment: Feces and Tissues.—1. Free from water by heating in a porcelain dish (Coors).

2. After the material begins to char, bring to a dull red heat and ash. Ashing must be very carefully conducted at a temperature well below full red heat, otherwise part or all of the lead will be lost by volatilization. Fecal material usually ashes readily, but the tissues form a residue which must be repeatedly extracted before the entire char is consumed. Usually the material requires re-ashing as a certain quantity of inorganic salts fuse and prevent complete oxidation.

Dissolve in hydrochloric acid diluted with an equal part of water and boil for about 20 minutes. Dilute with distilled water and filter while hot.

3. After the first ashing the material should be cooled. It is essential that *all* the ash be dissolved, for frequently lead phosphate is present as an insoluble residue which may be mistaken for silica. If this residue is insoluble in hydrochloric acid it should be treated with a mixture of hydrochloric and tartaric acids (which dissolves lead phosphate), until the ash is quantitatively dissolved. (Tartaric acid even of good quality usually contains lead and, therefore, should be tested with hydrogen sulphide.)

Urine.—1. Ammonium hydroxide is added to urine until it is strongly ammoniacal. This mixture is allowed to stand for an hour. In this reaction the earthy phosphates are precipitated and lead phosphate is carried down.

2. The gelatinous mass of phosphates settles into a compact mass from which the clear lead-free liquor may be decanted and the remainder rapidly filtered by suction on a Buchner funnel.

3. The filter paper containing the precipitate is ashed and the precipitate completely dissolved in dilute hydrochloric acid with the aid of heat if necessary. (The urine must be either freshly collected or well preserved with thymol, because crystalline phosphates which form when urine is allowed to become ammoniacal

on standing, do not completely remove the lead. Heating ammoniacal urine to increase the rate of settling of phosphates prevents complete recovery of lead.)

Procedure.—1. Carefully neutralize the hydrochloric acid solution with dilute sodium hydroxide using methyl orange as indicator. Add dilute hydrochloric acid until the solution is just acid to methyl orange.

2. Saturate the cold solution with washed hydrogen sulphide. If sulphides precipitate to any great extent during this process, they may be separated at once, but if no precipitate appears, the solution, saturated with hydrogen sulphide, should be allowed to stand overnight. Centrifuge and wash with boiled distilled water, three times altogether. (FeS is easily oxidized by the air to soluble $FeSO_4$.)

3. Dissolve the precipitate in nitric acid (3 to 5 c.c. concentrated). Boil to expel hydrogen sulphide, cool, and neutralize with dilute sodium hydroxide, using phenolphthalein as indicator.

4. Acidify with dilute acetic acid, and add an excess of potassium chromate —2 or 3 drops of a saturated solution. If the solution is held against a dark background during this process a slight turbidity may be observed around the drop of added chromate in the presence of even very minute quantities of lead. To hasten the reaction the solution should be boiled for a few minutes. If no turbidity is apparent the solution should be allowed to stand overnight. A yellow precipitate indicates the presence of lead.

5. If quantitative estimation is desired, proceed as follows: Filter; wash with warm water to remove all soluble chromate from the filter paper, and wash the precipitate completely into an Erlenmeyer flask. Wash the filter paper with 2 to 5 c.c. of 1:1 solution of hydrochloric acid followed by warm water, and collect in the same flask. The precipitate dissolves readily in hydrochloric acid. Add an excess of potassium iodide solution at once and titrate the liberated iodine with N/200 sodium thiosulphate, a drop or two of starch being added near the end-point as indicator:

1 c.c. N/200 sodium thiosulphate = 0.345 milligram lead

When only small amounts of lead (less than 1 milligram) are present, use a micro-buret graduated in 0.2 c.c. The sodium thiosulphate should be made up and preserved with suitable precautions for prevention of decomposition by carbon dioxide. Restandardize once a week.

METHOD FOR THE DETECTION OF METALLIC POISONS

Oxidation of Organic Matter (Fresinius von Babo's Method).—1. A portion of finely divided material is mixed with distilled water to a fluid mass and placed in a Kjeldahl flask.

2. About 30 c.c. of concentrated hydrochloric acid (arsenic-free if a test is to be made for arsenic) are added per 100 c.c. of material (a large excess of hydrochloric acid should be avoided).

3. Add 1 to 2 grams of potassium chlorate, shake well and set the flask upon

a boiling water bath under the hood. Nascent chlorine is evolved which destroys the organic matter. When the flask is hot, it is frequently shaken and a small amount of potassium chlorate (0.3 to 0.5 gram) is added from time to time until the solution is clear or turbid, has a pale yellow color, and additional heating produces no further change. Fat is very resistant to oxidation by chlorine.

4. When oxidation is complete, dilute with hot water and add a few drops of dilute sulphuric acid to precipitate possible barium; shake and pour the liquid through a wet filter paper.

5. Evaporate in a porcelain dish on a water bath nearly to dryness to remove excess acid. The decomposition of some potassium chlorate may give a brown color at this point. (If necessary, filter, wash with water, and evaporate again almost to dryness.)

6. Dissolve in water and filter. (The insoluble residues may contain silver chloride, barium sulphate and lead sulphate in addition to fat. These can be identified if necessary, after fusion with potassium nitrate and sodium carbonate.)

Nitric and sulphuric acids with a trace of potassium permanganate may be used as a substitute for oxidizing purposes.

Treatment with Hydrogen Sulphide.—The filtrate should have only a faint yellow color, and should be slightly acid (test with litmus). Place in a flask and heat on a water bath. While heating saturate the solution with washed hydrogen sulphide from a Kipp generator. (If a test is to be made for arsenic, the hydrogen sulphide must be arsenic free. Prepare arsenic-free hydrogen sulphide by saturating dilute sodium hydroxide solution with hydrogen sulphide from crude iron sulphide and commercial hydrochloric acid.) Pour this sodium hydrosulphide (NaSH) solution into a separating funnel and add slowly to dilute (1:4) sulphuric acid. Pass hydrogen sulphide into the hot solution for thirty minutes and continue for about thirty minutes after the flask has cooled, then stopper tightly and let stand for several hours, preferably overnight, and filter. The filtrate may contain chromium or zinc. The precipitate may contain arsenic, antimony, tin, mercury, lead, bismuth, copper, cadmium. Treatment with hydrogen sulphide almost invariably causes a precipitate of sulphuric and organic thio-compounds; therefore no positive conclusion can be drawn from the formation of precipitate at this stage.

Examination of the Precipitate.—The precipitate is thoroughly washed with hydrogen sulphide water and while still moist about 5 to 10 c.c. of a boiling solution of equal parts of ammonium hydroxide and yellow ammonium sulphide are dropped upon the precipitate on the filter. Repeat this several times. Finally wash with a few c.c. of a fresh mixture of ammonia and yellow ammonium sulphide. The filtrate may contain arsenic, antimony, tin, and copper (see Metallic Poisons I). The precipitate may contain mercury, lead, copper, bismuth, and cadmium (see Metallic Poisons II).

Metallic Poisons I.—Evaporate the solution (the solution is often dark brown owing to dissolved organic substances) to dryness in a porcelain dish on a water bath, cool, moisten with fuming or concentrated nitric acid and again evaporate to dryness. Then mix the residue with three times its volume of a mixture con-

taining 2 parts sodium nitrate and 1 part sodium carbonate. Thoroughly dry this mixture upon the water bath and introduce small portions at a time into a porcelain crucible containing a little fused sodium nitrate heated to redness. After the final addition heat the crucible a short time, introducing possibly a little more sodium nitrate until the fused mass is colorless. In the presence of copper the melt is gray or black from copper oxide. Sodium arsenate, sodium pyro-antimonate, sodium stannate as well as stannic oxide and copper oxide may be present. Soften the cold melt with hot water and wash into a flask. Add a little sodium bicarbonate to decompose the small quantity of sodium stannate possibly in solution and precipitate all the tin as stannic oxide and then filter. The filtrate contains any arsenic
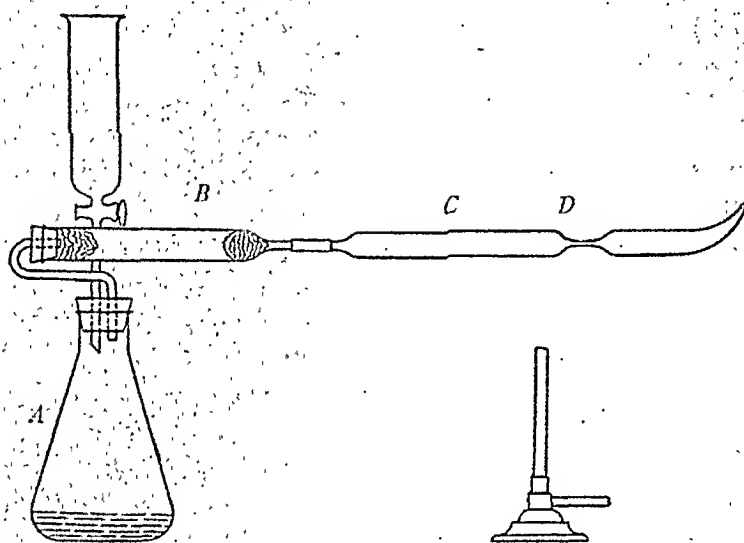
Fig. 369.—One Form of Marsh Apparatus Used in Testing for Arsenic

present as sodium arsenate and the residue may contain sodium pyro-antimonate, stannic and copper oxide.

Marsh Test for Arsenic.—Acidify the filtrate with arsenic-free sulphuric acid. Evaporate in a casserole over a free flame, and add sufficient arsenic-free sulphuric acid to expel nitric acid. Heat until copious white fumes of sulphuric acid appear. Arsenic if present is in the form of arsenic acid and is tested in the Marsh apparatus (Fig. 369).

Place about 30 grams of arsenic-free granulated zinc in flask *A*. *B* should be packed with anhydrous calcium chloride. Since pure acid invariably will not react with pure zinc it is necessary to add about 100 milligrams of copper sulphate, thereby forming an electric couple and causing hydrogen to be formed as soon as acid is added. Stopper tightly and pour 50 c.c. cold, dilute, arsenic-free sulphuric acid (1 volume concentrated sulphuric acid: 5 volumes water) into the funnel and regulate the flow of this acid upon the metal so that the hydrogen will not be generated too violently. The flask should be kept cool during the analysis by keeping it surrounded with cool water. If the temperature gets too high, sulphur

dioxide is formed and this in the presence of hydrogen is reduced to hydrogen sulphide (H_2S), which interferes with the test. All joints of the apparatus should be tight to avoid escape of arseniureted hydrogen (AsH_3), commonly called "Arsine" and also to prevent explosions. Air should be completely expelled before igniting to prevent explosion. To tell when this point is reached, collect hydrogen in a dry test tube until it ignites without detonation when carried to a flame. It may require as long as 30 minutes to expel the air. Test the hydrogen to insure its entire freedom from arsenic. Neither the arsenic mirror nor spot appear. With the hydrogen burning at the outlet, gradually introduce the perfectly cold sulphuric acid solution containing arsenic in small portions into the flask A by means of the same funnel. At the same time heat ignition tube C to redness just back of the constriction D. Keep reaction mixture cool by surrounding with cold water. Heat promotes the formation of hydrogen sulphide by reduction of sulphuric acid. Hydrogen sulphide is in turn decomposed on ignition to sulphur which may lead to the formation of a "false mirror." Also, if the stream of gas is too rapid, arsenic may be lost. If the solution contains arsenic, a shining metallic arsenic mirror is deposited just beyond the point of ignition. Antimony will give a mirror before and beyond the point of ignition. If organic substances are present a carbon mirror may be formed, and selenium will produce a yellowish-red or brownish-red mirror. If the flame is removed from C and a cold porcelain dish pressed down on the arsenic hydrogen flame at the tip, a brownish black spot is formed upon the dish. This spot dissolves readily in sodium hypochlorite solution (Dakin's). Antimony spots will not dissolve.

Extinguish the flame at the end of tube C and hold in the escaping gas a strip of filter paper moistened with concentrated silver nitrate solution (1:1). A yellow stain appears if the hydrogen contains arsenic and a drop of water. (See note regarding presence of "normal arsenic" under Reinsch test.)

Metallic Poisons II.—Over the substance on the filter paper remaining after the treatment with the hot ammonium sulphide mixture there are poured several small amounts of warm, rather dilute nitric acid (1 volume concentrated nitric acid: 2 volumes water). Mercuric sulphide does not dissolve but all the other sulphides pass into solutions as nitrates. The filtrate may contain lead, copper, bismuth, and cadmium, and specific tests are available for their recognition. (If the filtrate contains lead, dilute sulphuric acid produces a white precipitate of lead sulphate.)

TEST FOR MERCURY.—Treat the substance remaining on the filter paper after nitric acid treatment, even though not black, with a little hot dilute hydrochloric acid containing a crystal of potassium chlorate, and pass the acid through the paper several times. Evaporate the filtrate to dryness in a porcelain dish upon the water bath and dissolve in 5 c.c. of 5% hydrochloric acid, filter and test the filtrate for mercury as follows:

(a) *Stannous Chloride Test.*—Add to a portion of the filtrate a few drops of stannous chloride solution. A white precipitate of mercurous chloride appears if

mercury is present. Excess of stannous chloride, especially if heat is applied, reduces this precipitate to gray metallic mercury.

(*b*) *Copper Test.*—Put a few copper slugs (previously cleaned in concentrated nitric acid and washed) into a portion of filtrate. Mercury deposits as a gray spot on standing or on heating. Wash the slugs successively in water, alcohol, and ether. Dry thoroughly and heat in a small bulb-tube of hard glass. Mercury sublimes and collects in small metallic globules on the cold sides of the tube. A trace of iodine vapor introduced into the tube immediately transforms the gray sublimate into scarlet mercuric iodide.

The following outline shows the general plan of the above system of analysis.

Material: Treated with hydrochloric acid and potassium chlorate. Dilute sulphuric acid. Filter.		
Filtrate: Saturate with warm hydrogen sulphide.		Precipitate tested for silver, lead, barium.
Precipitate: Treated with hot ammonium sulphide and ammonium hydroxide.	Filtrate tested for chromium, zinc.	
Filtrate tested for arsenic, antimony, tin, copper. (Metallic Poisons I.)	Precipitate tested for mercury, lead, bismuth, copper, cadmium. (Metallic Poisons II.)	

METHODS FOR THE DETECTION OF CARBON MONOXIDE IN THE BLOOD

Tannic Acid Test.—Prepare a solution of 1 part of blood with 4 parts of distilled water. Add 3 volumes of a 1% solution of tannic acid solution and shake well. Normal blood is gray but in the presence of carbon monoxide the blood gives a cherry-red (carmine red) color.

Pyrogallic-Tannic Acid Test.—1. Procure about 10 c.c. of normal blood which has been prevented from clotting by the use of an anticoagulant (potassium or sodium citrate is the anticoagulant of choice) in the proportion of 0.05 gram per 10 c.c. of blood.

2. Pipet 3 c.c. of blood into a 50 c.c. graduated cylinder and dilute with distilled water to the 30 c.c. mark.

3. Saturate the rest of the blood with 3 to 5% carbon monoxide gas ("illuminating gas" may be used) and then dilute 3 c.c. to a volume of 30 c.c. with distilled water.

4. From these solutions of oxyhemoglobin and carbon monoxide hemoglobin, mixtures are made which vary from 0 to 100% in steps of 10 as follows:

Two c.c. amounts of these solutions are placed in small test tubes of uniform bore.

5. To each standard thus prepared are added 2 c.c. of strictly fresh pyrogallic-tannic acid solution and the tubes are inverted twice to insure mixing. Do not shake.

6. The tubes should be sealed immediately by pouring a little melted paraffin on top of the contents while the tube is immersed in cold water as a caution against overheating.

Per Cent	Carbon Monoxide Hemoglobin, c.c.	Oxyhemoglobin, c.c.
100	2	0
90	4.5	0.5
80	4	1
70	3.5	1.5
60	3	2
50	1	1
40	2	3
30	1.5	3.5
20	1	4
10	0.5	4.5
0	0	2

Procedure (Sayers and Yant).—1. Dilute 0.5 or 1 c.c. of the whole blood to be analyzed, prevented from clotting by the addition of an anticoagulant, 1:10 with distilled water.

2. Pipet 2 c.c. of this diluted blood into a test tube uniform in bore with those of the standards and add with a pipet 2 c.c. of strictly fresh pyrogallic-tannic acid solution. Invert the tube several times to insure mixing. Do not shake!

REAGENT

Tannic acid, C. P. (2% sol.).........................25 c.c.
Pyrogallic acid, C. P. (2% sol.).....................25 c.c.
To be made up fresh.

3. Let stand 15 minutes and read against the standards. If carbon monoxide is present, let stand 15 minutes longer, read and report the latter reading as "per cent blood saturation with carbon monoxide."

Notes.—1. In carbon monoxide bloods prevented from clotting by oxalate, there is an appreciable change of carbon monoxide to carbon dioxide on standing. This is not true when citrate or sodium fluoride are used.

2. When *saturating blood with carbon monoxide*, in order to prevent loss of blood due to excessive foaming, the operation be carried out in a beaker with constant stirring.

3. The blood for the standards should be saturated with carbon monoxide before it is diluted with distilled water in order to minimize the volume of carbon monoxide gas physically dissolved in the resulting solution.

4. If it is desired to preserve the standards, air must be excluded. The tube walls above the paraffin should be thoroughly dried and a permanent seal made by placing a disk of cardboard on top of the paraffin and filling the remainder of the tube with ordinary sealing wax. Standards thus prepared and kept in a cool place will retain their permanence for one to two weeks, not changing enough to interfere with the accuracy of the determination. Permanent standards prepared from artists' oil colors are on the market.

METHOD FOR THE DETECTION OF CYANIDE

Principle.—The principles involved are clearly indicated under the various procedures outlined. Great care must be exercised in carrying them out because of the dangers to the analyst and secondly the interfering substances.

Organs Best Suited for Analysis.—If the poison has been taken by mouth, the stomach contents and brain should be analyzed. Analysis of the brain is necessary for the purpose of ruling out the possibility of the poison having been introduced into the stomach after death. If the poisoning resulted from inhalation, the lungs and brain must be examined. In cases of poisoning by inhalation, usually none, or only the very faintest trace, is found in the stomach contents. This is of tremendous importance from the medicolegal aspect.

Method of Isolation of Cyanide.—The tissues are cooled by keeping them in an ice-box. Two hundred to 500 grams of tissue are ground up. Care should be taken to keep the tissue cold since hydrocyanic acid may volatilize if warm. If stomach contents are analyzed, usually one-fifth of the total volume of the contents is used. The ground-up tissue or the stomach contents are placed in a one liter flask and acidified with tartaric acid. The material is then distilled with steam, using a well cooled condenser the tip of which is bent to serve as an adapter and dipped into 5 c.c. of 5% sodium hydroxide solution in a receiving flask. The latter should be packed in ice. One hundred cubic centimeters of distillate are collected, which is ample to recover all the cyanide present, the following tests being employed.

Qualitative Tests.—1. *Schönbein's Test.*—Suspend a strip of filter paper, impregnated with guaiac and copper sulphate, over the material in a flask, the paper being held in place by the stopper. (Dip strip of filter paper into a freshly prepared alcoholic solution of guaiac 1:10; then let dry; when dry, moisten it with dilute (1:10.000) copper sulphate solution). If color does not change, cyanide is absent and no further tests need be made. If a blue color results, cyanide *may* be present. The test is very sensitive but not specific; hydrochloric acid, nitric acid, chlorine, bromine, ozone, hydrogen peroxide, as well as some other substances also give a positive test. This test may be used as a preliminary one at the necropsy table. The following two tests must be employed since they are specific for cyanide:

2. *Prussian Blue Test.*—To 5 c.c. of distillate, add 3 c.c. of 25% sodium hydroxide. then a few drops of freshly prepared ferrous sulphate solution and a few drops of ferric chloride solution. Warm a little. Let cool and add concentrated hydrochloric acid, drop-wise, until the dirty brown precipitate just dissolves; avoid excess hydrochloric acid. If cyanide is present, a deep blue precipitate (Prussian blue) appears. If only a trace of cyanide is present, a green solution results instead of a blue precipitate, but, on standing several hours, a small flocculent Prussian blue precipitate settles (sensitive to one part in 50,000).

3. *Liebig's Test.*—To 10 c.c. of distillate, add 1 c.c. of yellow ammonium sulphide and evaporate to dryness on the water bath. When dry, add 5 c.c. of 5% hydrochloric acid solution, warm a little and stir well to dissolve all of

the thiocyanate that was formed during the evaporation. Let stand 2 hours, then filter. To the filtrate, add 5 to 10 drops of 10% ferric chloride solution. If cyanide is present, a deep red color results. (This test is sensitive to one part in ten million.)

The following tests may be used, but they are not specific for cyanide:

4. *Vortmann's Test.*—To 5 c.c. of distillate, add a few drops of potassium nitrate solution, then 2 to 4 drops of ferric chloride solution and then enough dilute sulphuric acid until the color of the solution becomes a bright yellow. The solution should then be boiled, after which cool and add ammonium hydroxide until all of the iron is precipitated. Filter off the precipitate. To the filtrate add a few drops of a very dilute solution of ammonium sulphide. If cyanide is present, a play of colors results, violet, blue, green, yellow.

5. *Picric Acid Test.*—To 5 c.c. of distillate (slightly alkaline) add a few drops of picric acid solution and warm gently. If cyanide is present, a red color develops (sensitive to one part in one million).

6. *Phenolphthalein Test.*—To 5 c.c. of distillate, add a few drops of alkaline phenolphthalein solution (reduced phenolphthalein), then a few drops of 1:2000 copper sulphate solution. If cyanide is present, a red color develops (sensitive to one part in 20 million).

7. *Silver Test.*—To 2 c.c. of distillate, add nitric acid until reaction is acid, then add a few drops of silver nitrate solution. If cyanide is present, a white precipitate of silver cyanide results.

Quantitative Test.—For quantitative analysis, a weighed amount of tissue is distilled, as described in the qualitative procedure. In the receiving flask, however, instead of having dilute sodium hydroxide, 20 c.c. of 10% silver nitrate solution are used, acidified with nitric acid. In order to make certain that all of the cyanide is isolated, distillation is continued until 200 c.c. of distillate are obtained. During the distillation, the cyanide precipitates as silver cyanide. This is then filtered through a previously weighed Gooch crucible, washed, dried and weighed. From the weight of the silver cyanide, the amount of cyanide in the material analyzed is calculated as HCN.

In quantitative analysis, if the poison was taken by mouth, the entire amount of cyanide is determined in the gastro-intestinal tract. This is then multiplied by 100/98, which gives the amount in the entire body. This fraction is used because approximately 98 per cent of the cyanide, if taken by ingestion, remains within the stomach contents. If the poison was introduced by inhalation or injection, parts of all the organs and tissues are analyzed and, from these results, the amount present in the entire body is calculated.

The lethal dose of cyanide is accepted as 50 mgm., calculated as hydrocyanic acid.

The following factors interfere with the determination of the presence of cyanide:

1. Traces of hydrogen cyanide are produced during the first few days of putrefaction, but this disappears in the later stages.

2. Cyanides present in the tissues disappear ... and are changed to sulphocyanide.

3. In stomach contents, where the bulk of the ... faction is of little importance.

4. Embalming with formaldehyde interferes greatly ... forming condensation products with the formaldehyde.

It is, therefore, important that early toxicological ... of suspected cyanide poisoning. It is likewise necessary ... of ferrocyanides, ferricyanides and thiocyanates before ... cause these compounds, when distilled in the presence of ... hydrogen cyanide.

Diffusion of the poison in a body after death has been ... process is extremely slow and it has been shown by G... is introduced into the stomach after death, no cyanide rea... an interval of two months.

Although the term "cyanide of potassium" is generally ... to cyanide poisoning, it is not the potassium salt but, because ... the sodium salt that is used.

METHOD FOR THE DETECTION OF BORIC ACID

The specimen of urine, stomach washing or finely divid... strongly alkaline with a solution of sodium hydroxide or sodium ... evaporated to dryness on the water bath. It is then carefully ... heat. The ash is acidified with concentrated HCl, and divided ... To one portion ethyl alcohol is added, and then ignited. A green ... boron. This flame examined with the spectroscope gives four chara... tion bands in the yellow, green, and blue green. The second por... with a little water and tested with turmeric paper. If boric acid ... paper turns a red brown, which is intensified on drying. In acute ... mouth it is possible to determine whether boric acid or borax ... analyzing the stomach contents or stomach washing. The internal or... urine are useless for this purpose. If the stomach washing is very ... indicates borax; further, if the stomach contents are first dried on ... bath, ethyl alcohol will extract boric acid, but not borax. If it is desired to ... the amount of boron present, the material made alkaline with Na_2CO_3 ... and ashed at low red heat. The ash is then thoroughly extracted with dil... hydrochloric acid, transferred to a volumetric flask and water added to ... volume. To 10 c.c. of this solution is added some turmeric solution and ... that is produced is compared with that of a standard boric acid solution ... colorized with turmeric solution.

METHOD FOR THE DETECTION OF SODIUM CARBONATE

(*Washing Soda*)

The stomach contents, or the stomach washings (with water) must be used in analyzing for sodium carbonate. Due to the presence of Na and CO_3 ions in the blood and tissues normally, and also due to the buffer action of the blood and tissue fluids, it is impossible to definitely establish whether any exogenous sodium carbonate is present in the blood, tissues or urine. The stomach washings can be tested directly, unless a large amount of food is present. If so, the stomach contents should be dialyzed, and the resulting dialyzate used for the tests. A strip of litmus paper is introduced in order to ascertain whether the material is at all alkaline. If so, a few drops of phenolphthalein solution are added to some of the material. A deep red color is produced if sodium carbonate or sodium hydroxide is present. Sodium bicarbonate will give only a faint pink. To differentiate between sodium hydroxide and sodium carbonate, hydrochloric acid is added to some of the material. The liberation of much gas (CO_2) indicates carbonate. For the quantitative estimation, a known excess of N/20 sulphuric acid is added to a measured amount of the diluted and filtered stomach contents, then boiled one to two minutes, cooled, and the sulphuric acid remaining is titrated with N/20 NaOH. From the amount of sulphuric acid neutralized by the stomach contents, the amount of Na_2CO_3 is calculated.

METHOD FOR THE DETECTION OF POTASSIUM CHLORATE

The stomach contents, urine, blood, or finely divided tissues are placed into a dialyzing bag made of collodion or parchment paper. Dialysis into pure water is continued for several hours. To some of the dialysate is added an excess of silver nitrate solution. The resulting precipitate of silver chloride is filtered off. The clear filtrate is acidified with nitric acid and sulphurous acid added, and the mixture is then brought to boiling. If a white precipitate remains it is silver chloride from the reduction of the chlorate by the sulphurous acid.

The simplest method for the quantitative estimation of the chlorate is to determine the chloride content of an aliquot portion of the material by one of the usual methods. A second measured portion of the material is made strongly alkaline with added sodium carbonate, well mixed, dried, and carefully ignited at low red heat. This procedure converts the chlorate into chloride. The chloride content is now determined in this ignited material. From the difference between the two chloride determinations, the amount of chlorate originally present can be calculated.

METHOD FOR THE DETECTION OF PHOSPHORUS

In acute (white) phosphorus poisoning, the gastro-intestinal contents is of prime importance from an analytical standpoint. The material is distilled in a dark room screening off the light from the Bunsen burner. If white phosphorus is present, a luminescent ring will be noticed during the distillation, in the upper part of the

water cooled condenser. For a further test, the distillate is strongly acidified with concentrated nitric acid and then evaporated to dryness on the water bath. This procedure converts the phosphorus to phosphoric acid, which can then be identified by the ammonium molybdate reaction.

METHOD FOR THE DETECTION OF ANILINE AND NITROBENZENE

For the detection of aniline and nitrobenzene. the finely divided organs, urine. blood, or stomach contents are distilled with steam. If these substances are present in appreciable amount, the distillate is usually cloudy. To one portion of the distillate add two drops of chloroform and excess NaOH solution, and bring to boiling. If aniline is present, the very irritating and piercing odor of phenyl isocyanide develops. Should nitrobenzene be present, the steam distillate is shaken out with ether. The ether layer is allowed to evaporate spontaneously. If nitrobenzene was present, it will now be found in the form of a few oily globules with the characteristic odor of oil of bitter almonds. These drops are dissolved in alcohol. The alcoholic solution is reduced with powdered zinc and HCl. This converts the nitrobenzene to aniline. After making the solution alkaline with NaOH, the aniline obtained is tested by means of the isonitrile reaction as above.

METHOD FOR THE DETECTION OF CERTAIN ORGANIC VOLATILE POISONS
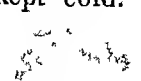
For the detection of benzene, chloroform, ethyl-chloride, ether, carbon-bisulphide, carbon-tetrachloride and others of the many new volatile solvents and anesthetics considerable apparatus and technical skill are required which are hardly applicable in the average clinical laboratory. The methods are described in detail by Gettler and Siegel (Isolation from Human Tissues of Easily Volatile Organic Liquids and Their Identification. *Arch. Path.*, 1935. 19:208-212).

Nearly all these compounds have a characteristic odor which can be readily identified in the patient's breath or vomitus or in the stomach washings. Great care should be taken of vomitus or gastric washings to prevent the rapid evaporation of these poisons. The material must be placed in a tightly stoppered container and kept in the refrigerator. Frequently a simple distillation of the material on a water-bath, but using an electric plate for heating (to prevent ignition or explosion of some of these compounds) will bring out the characteristic odor. A long condenser. preferably cooled with ice-water, is desirable.

METHOD FOR THE DETECTION OF WOOD ALCOHOL (METHANOL)

1. The material (vomitus. tissue, etc., or the contents of the suspected liquid) is acidified with tartaric acid and enough distilled water is added to make it fluid (to a volume of 100 c.c.) and then steam distilled.

2. Collect 50 c.c. of distillate, stopper and shake. Of this distillate 10 c.c. is taken in a test tube and a heated copper spiral is plunged into the liquid. This is repeated 8 times and the test tube is held under cold running water. This converts the wood alcohol into formaldehyde. The test tube must be kept cold.

Then 2 c.c. of this oxidized liquid is poured into another test tube and the following test is made:

    (a) Add 10 drops of a 5% sodium phenylhydrazine hydrochloride solution
    (b) Add one drop of a 0.5% sodium nitroprusside solution
    (c) Add 3 drops of a 10% sodium hydroxide solution

A blue color develops if formaldehyde is present—and indicates that wood alcohol (methanol) is present. The intensity of the blue color gives a rough estimate of the amount present.

A red color, with the above reagents indicates acetaldehyde which is obtained in the manner described above when grain (ethyl) alcohol is present. For further tests see A. O. Gettler (Critical Study of Methods for the Detection of Methyl Alcohol, *J. Biol. Chem.*, 1920, 42, 2:311, 328).

METHOD FOR THE DETECTION OF GRAIN (ETHYL) ALCOHOL

Much has been written concerning the detection of ethyl alcohol, especially in the presence of other alcohols, and also its medicolegal significance in intoxication. It has been fairly well established that the various methods to determine intoxication, such as an estimation of the amount found in the urine or expired air, are valueless, since they are excreted and no longer causing the inebriation. (Inebriation depends upon the amount of unoxidized alcohol in the brain.) Hence the ideal method is to determine the amount of alcohol present in the brain tissue. This of course is easy in the cases coming to necropsy, but correspondingly difficult in an intoxicated individual. In such cases examination of the spinal fluid is probably of greatest value. For further details the reader must consult the enormous literature which has accumulated. (See references.)

METHOD FOR THE QUALITATIVE DETERMINATION OF GRAIN- (ETHYL) ALCOHOL

For an ordinary rapid method which is applicable to urine, blood, spinal fluid or finely divided tissue, the method of oxidation acid testing is carried out exactly as described under wood alcohol (methanol) and the red color obtained indicates the presence of ethyl alcohol. Large amounts of urine and blood are necessary for these determinations.

METHOD FOR THE QUANTITATIVE DETERMINATION OF ETHYL ALCOHOL IN TISSUES

Principle.—Depends upon the oxidation of ethyl alcohol to acetic acid and titrating with N/20 sodium hydroxide.

1. The tissue, as soon as it is removed from the body, should be placed in a tightly closed jar and placed in a refrigerator. When ice cold, 500 gms. are weighed out, quickly ground up and placed in a 2 liter flask. To this are added 600 c.c. of water, 5 c.c. of a saturated solution of tartaric acid and 1 c.c. of white mineral oil. This mixture is now distilled with steam. A long, well cooled

condenser should be used, and the distillation should be continued until exactly 800 c.c. has been collected. The distillate is well mixed and used in the following procedure.

2. Twenty grams of potassium dichromate and 40 c.c. of concentrated sulphuric acid are placed in a 500 c.c. distilling flask. Three hundred cubic centimeters of the distillate obtained above is now added and the contents mixed well. The flask is then connected to a long, well cooled condenser by means of a Hopkins distilling head, and the distillation started. The heat must be so regulated that it will take from forty-five to fifty minutes to collect exactly 250 c.c. of distillate. After thoroughly mixing, 50 c.c. of this distillate is titrated with twentieth normal sodium hydroxide solution, using phenolphthalein as an indicator. From this titration figure, the amount of alcohol present in 1 kilogram of brain tissue can easily be calculated as follows: (c.c. twentieth normal alkali − 1.32) × 71.58 = mg. C_2H_5OH per kilogram of tissue. The figure (−1.32) holds only for brain tissue. For other tissues a fair average would be calculated as follows:

$$1 \text{ c.c. } \frac{N}{20}NaOH = 2.3 \text{ mgms. of ethyl alcohol.}$$

The following rules have been laid down by Gettler and Tiber:

1. For the qualitative and quantitative determination of alcohol, the brain is of first importance.

2. The normal alcoholic content of human brain material is less than 0.0025%.

3. The alcoholic content of the brain in persons who have partaken of alcoholic beverage ranges between 0.005 and 0.6%.

4. All patients having an alcoholic content below 0.1% show no abnormal physiologic effects.

5. Patients with an alcoholic content above 0.1% and up to 0.25% show some physiologic disturbance, as evidenced by increased aggressiveness, and more or less loss of the sense of care. None of these patients, however, shows unbalanced equilibrium, which is commonly called intoxication.

6. When the alcoholic content rises above 0.25% and up to 0.4% and still higher to 0.6%, the equilibrium of the person becomes unbalanced—a condition generally known as intoxication.

7. The degree to which any person is effected does not depend on the quantity of alcohol consumed, but on the amount of alcohol present in the brain at the time.

METHODS FOR THE DETECTION OF VERONAL, LUMINAL, MORPHINE, STRYCHNINE, BRUCINE, CODEINE AND COCAINE

Principle.—The above substances are separated by extraction, purified, and identified by one or more of the tests listed under each individual substance.

The procedure which follows is devised to identify one or more of the above when present in human organs, meat, vegetables, milk, potatoes and other substances.

Preparation of Material for Extraction.—1. Mix a quantity of finely ground material in a large flask and mix with 2-3 times as much absolute alcohol. Add enough tartaric acid (10%) to make the mixture acid to litmus. Avoid a large excess of tartaric acid.

Using a Liebig condenser as a reflux, heat the flask on a water-bath for 15 or 20 minutes at 60° C.

2. Cool the flask and contents and filter off as much fat as possible. Rinse the residue with alcohol, filter and combine the filtrates. Should the filtrate fail to be acid, acidify with tartaric acid.

3. Evaporate the filtrate to a thin syrup on a water-bath and mix the residue with 100 c.c. of cold water.

4. Filter through moistened filter paper and evaporate to a syrup on a water-bath.

5. Mix the residue thoroughly with liberal quantities of absolute alcohol. Filter. Evaporate the filtrate on a water-bath and dissolve the residue in about 50 c.c. of water. Should this solution fail to be perfectly clear, filter through moistened filter paper. This filtrate (or original solution) should have an acid reaction. For convenience in future reference to this solution, it is designated as "Solution A."

Extraction and Identification of Veronal and Luminal.—1. Extract "Solution A" several times with ether. Evaporate the ether extract to dryness. (Save acid aqueous solution for possible tests described below.)

2. Recrystallize the ether extract residue from a small quantity of hot water, using animal charcoal to remove color.

3. The identification of veronal and luminal can be made on the basis of the following properties:

(a) *Color Reaction.*—This test identifies the barbiturates as a group, and is not specific for veronal or luminal. To each of 3 tubes (No. 1, No. 2, No. 3), add some chloroform solution of the suspected purified crystals. To each add 1-2 drops cobalt acetate (1%) in methyl alcohol. To tube No. 1 add 2 drops of barium hydroxide in methyl alcohol; to tube No. 2 add 4 drops and to tube No. 3 add 8 drops. A blue color in any of these tubes constitutes a positive test for the barbital group.

(b) *Melting-Point.*—This determination is important because it serves as practically the only reliable means of differentiating between individual barbiturates.

The melting-point of pure veronal is 188-191° C. When impure it melts at 187-188° C.

The melting-point of luminal is 171-173° C.

(c) *Sublimation.*—Veronal when heated cautiously, easily sublimes.

Test for Veronal in Urine.—1. Take about 500 c.c. of urine for analysis.

2. Make strongly acid with tartaric acid and evaporate in a porcelain dish to a thin syrup on a water-bath.

3. Mix the syrup with 100-150 c.c. of alcohol; filter and evaporate to dryness on a water-bath.

4. Dissolve the residue in about 60 c.c. of water and extract 2-3 times with 50 c.c. portions of ether.

5. Permit the ether extracts to settle, filter through dry filter-paper and distill. Veronal when present usually solidifies to a crystalline mass.

6. Dissolve in cold sodium carbonate solution (1:10), decolorize with animal charcoal, filter and precipitate with dilute hydrochloric acid. Apply the tests already described to the purified yield.

Extraction and Identification of Morphine.—1. "Solution A" is made strongly alkaline with sodium hydroxide solution and extracted with ether. Most alkaloids, except morphine, are in the ether extract. Save the ether extract for possible tests under IV.

2. Make the alkaline aqueous solution acid with dilute hydrochloric acid and then make alkaline with sodium bicarbonate.

3. Morphine is extracted with a hot solution consisting of 9 volumes chloroform and 1 volume absolute alcohol. (Method of Kippenberger).

4. Pour the combined chloroform extracts into a dry vessel, add a few crystals of anhydrous sodium sulphate to remove any water which may be present and filter the chloroform extract (dry filter) into a small vessel. Evaporate to dryness.

5. Purification: The residue is dissolved in hot amyl alcohol and shaken thoroughly with water acidified with dilute sulphuric acid. The aqueous extract is then made alkaline with ammonia, and extracted several times with hot chloroform. Evaporate to dryness.

(a) Morphine appears as white crystals, the melting-point of which is 230° C.

(b) Morphine is strongly alkaline and can be titrated.

(c) With Mandeline's reagent the color changes are red violet, to blue, to violet.

(d) With Froehde reagent the color changes are violet, to blue, to green.

(e) *Physiological test:* A neutral solution of morphine (HCl) when injected under the dorsal skin of a white mouse causes the tail of the animal to become S shaped. Five hundredths of a milligram of morphine will maintain the tail in this position for 45 minutes. Five milligrams will maintain it for 20 hours.

Extraction and Identification of Strychnine, Brucine, and Codeine.— "Solution A" is made strongly alkaline with sodium hydroxide solution and extracted 3-4 times with ether. Set the combined extracts aside in a dry flask (loosely stoppered) for 2-3 hours. Decant through a dry filter and evaporate from a small dish on a water-bath which has been previously warmed. The residue will contain *Strychnine, Brucine*, and *Codeine*.

Identification of Strychnine.—1. Crystals melt at 268° C.

2. With Mandeline's reagent the color changes are blue to blue violet to red violet to red, and in 15-20 min. to orange. If the orange color fails to appear Strychnine *is not* present.

3. Erdmann's and Frochde's reagents give no color change with strychnine.

Identification of Brucine.—1. Color changes using Erdmann's and Froehde's reagents may be employed to identify Brucine.

2. Erdmann's reagent gives a red color which changes to yellow.

3. Froehde's reagent also gives a red color which changes to yellow.

Identification of Strychnine in the Presence of Brucine.—1. Brucine when present together with strychnine may render inconclusive the tests for the latter. To avoid this the brucine can be destroyed before applying the strychnine tests.

2. The residue (or part of) containing brucine is dissolved in about 2 c.c. of dilute sulphuric acid, 2 drops of concentrated nitric acid are added, and the system permitted to stand for 2-3 hours. It is then made strongly alkaline with sodium hydroxide solution and extracted with ether. The evaporated ether extract will contain strychnine but not brucine which is destroyed by this procedure.

Method for the Identification of Codeine.—1. When codeine is warmed with Erdmann's reagent a blue color results.

2. With Froehde's reagent there is obtained a yellow green color which changes to blue.

3. Mandeline's reagent with codeine gives a green color which changes to blue.

Method for Differentiation between Morphine and Codeine.—1. On the addition of an aqueous solution of ferric chloride: A blue color is obtained with *morphine.* No change takes place in the case of *codeine.*

2. On the addition of hydrogen iodide: Iodine is rapidly liberated in the case of *morphine.* No iodine is liberated in the case of *codeine.*

Method for the Extraction and Identification of Cocaine.—"Solution A" is made alkaline with sodium bicarbonate solution and extracted with ether. Evaporate to dryness and apply the following tests for the identification of cocaine:

1. When cocaine is warmed with Marquis reagent, a wine red color is obtained.

2. Add a small amount of sulphuric acid (conc.), and a crystal of potassium iodate to the suspected cocaine. At this point there is no visible change but when the solution is heated until SO_3 fumes appear the following color changes take place: Brown, to olive, to blue, to violet, which finally fades.

3. Add a small amount of sulphuric acid (conc.) and place on a water-bath for 5 minutes. Then carefully add water. If cocaine is present there is obtained the odor of methyl benzoate (Oil of Peppermint).

SPECIAL REAGENTS USED ABOVE

Erdmann's Reagent.—Add 10 drops of a mixture of 10 drops of concentrated nitric acid and 100 c.c. of water, to 20 c.c. of concentrated sulphuric acid.

Froehde's Reagent.—Dissolve (by gentle heating) 5 mg. of molybdic acid or sodium molybdate in .1 c.c. of concentrated sulphuric acid. This solution should be colorless. It does not keep well.

Mandeline's Reagent.—Dissolve 1 part of finely ground ammonium vanadate in 200 parts of concentrated sulphuric acid.

Marquis Reagent.—Mix 2-3 drops of 40% formaldehyde solution with 3 c.c. of concentrated sulphuric acid. Prepare this solution when needed.

METHOD FOR THE DETECTION OF BROMINE

Principle.—Urine is ashed with alkali. From the resultant bromides, bromine is liberated and finally concentrated in chloroform, with the production of a brown color.

Procedure.—1. In a nickel crucible place about 50 c.c. of the suspected urine. Add 1 gm. of sodium carbonate and 0.5-1 gm. of potassium nitrate and evaporate to dryness. (The capacity of the crucible employed should be approximately twice the volume of the sample taken for analysis.)

Remove adherent particles of carbon by sprinkling powdered potassium nitrate over the dried residue. During this latter operation rotate the crucible and continue heating.

2. Permit the system to cool to room temperature, add 10-15 c.c. water to the ash, and make distinctly acid (to litmus) with dilute sulphuric acid. The solution at this point is, as a rule, turbid.

3. Transfer to a separatory funnel, rinsing the crucible with 10-20 c.c. of acid potassium sulphate (10%).

4. Add 20-30 c.c. of chloroform, followed by a drop by drop addition of potassium permanganate (3%) until the color no longer disappears. Finally an excess of 15 drops of permanganate is added.

5. Stopper the cylinder and place in the dark for 15 min. Shake well and permit the brown solution of bromine in chloroform to settle.

TEISCHMANN'S TEST FOR BLOOD STAINS

Principle.—The test depends on the fact that hematin is formed from the decomposition of the hemoglobin by heat, and secondly, that the hematin in solution in boiling glacial acetic acid unites with the chlorine of the salt to form chloride of hematin, which is soluble in boiling glacial acetic acid, crystallizing from this solvent on cooling.

Procedure.—If the material is a dried blood stain, a small fragment of the dried blood should be removed from the stain with the point of a knife and transferred to a glass slide. If the stain be a diffused one, or if the blood, while still fresh, has soaked into the fabric, as in the case of a stain on cotton or linen cloth, then it suffices to scrape a small portion of the stain with the knife point, collecting the dust thus removed on a glass slide. The fragment of dried blood or the dust should then be treated on the slide with a small drop of water in which has been dissolved a minute fragment of sodium chloride. This drop should then be evaporated to dryness by gentle heat. the dried residue covered with a cover glass, a drop of glacial acetic acid allowed to run under the cover glass, and the slide again gently heated until bubbles of gas are seen to form in the liquid under the cover glass. This shows that the glacial acetic acid has been heated to the boiling point. If, now, the slide be allowed to cool, the microscope will reveal the characteristic crystals of chloride of hematin in case the stain

examined contained blood. These crystals of chloride of hematin are called "hemin" crystals, and they have a characteristic form (Fig. 370).

The normal hemin crystals have a yellow to chocolate-brown color and separate in the form of small rhombic plates. They naturally vary a little in size according to the rapidity of their formation. Sometimes, particularly if the fragment of dried blood on the slide was of considerable size, the form of the crystals in some parts of the preparation may be somewhat modified, some assuming a
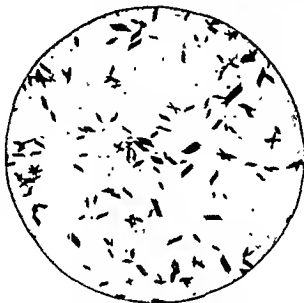
Fig. 370.—Hemin Crystals (Wood)

pointed, oval shape, and in some the outlines may be a little irregular; in all cases, however, a sufficient number of the normal perfect crystals will be seen to render their identification positive.

Precautions.—1. Care should be taken in heating the slide not to raise the temperature so high as to decompose the hematin in the first dry residue obtained. If the temperature be raised to about 142° C. (287.6° F.), no hemin crystals will be formed.

2. On further heating the slide, after the addition of glacial acetic acid, the temperature should not be raised so high as to produce active boiling of the acid, since active ebullition may carry all the pigment beyond the edge of the cover glass, which might prevent the detection of the hemin crystals.

3. The hemin test will not detect blood pigment in blood stains that have been heated to a high temperature, that have been subjected to the prolonged action of naphtha, solution of ammonium chloride, or bromochloralum, or that have been exposed for a long time to direct sunlight.

4. This is by far the most important test for blood pigment, and it is extremely delicate. While the detection of hemoglobin shows with certainty the presence of

blood, it throws no light upon the nature of the animal from which the blood came. To determine this latter question resort is had to microscopic examination of the blood cells and especially to the complement-fixation and precipitin tests.

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CHAPTER XXXVIII

HISTOLOGICAL METHODS AND THE PREPARATION OF MUSEUM SPECIMENS

FRANK W. KONZELMANN

Histologic or tissue technic is an art that is mastered only by patience and experience. It is the one field in pathology wherein these two qualities and the willingness to strive for perfection are of paramount importance. Accuracy in pathologic diagnosis requires perfectly prepared tissue sections as well as a broad experience and a knowledge of the principles of pathology. It cannot be hoped for with either alone, yet one observes that the most experienced pathologist is the most exacting in matters of technic while the least experienced is often satisfied with preparations of the poorest quality.

EQUIPMENT

Apparatus of the best grade, properly cared for and reagents carefully prepared are prerequisites of good technic.

The chief apparatus of the histologie technician consists of the microtome and its accessories, the paraffin oven, staining dishes and jars, and the microscope.

The Microtome.—There are many types. The Spencer rotary microtome is the best for paraffin embedded tissue. The microtome should never be taken apart except by a mechanic trained in this field. It should, however, be frequently cleaned by wiping all the accessible moving parts with a cloth moistened with xylol and well lubricated by a good grade of paraffin oil furnished by the manufacturer. Each day after it has been used, it should be cleaned of paraffin (a good stiff-haired one inch painter's brush is handy for this purpose). All water must be removed from the crevices of the knife holder and chassis. After it is thoroughly dried, a little "Three in One" oil may be rubbed over the exposed parts to prevent rusting. The instrument should be covered with a bag or bell jar.

The sliding microtome may be used for paraffin sections, especially for those greater than 2 cms. in diameter. The knife must be set so that its long axis forms a true right angle with the direction of its cutting motion. It must be parallel, in other words, with the trimmed edge of the paraffin block. Those types in which the knife is supported at both ends are the best. The knife is held by a carriage which rides upon a well-lubricated track, while the tissue block remains stationary. This is the best type of microtome for celloidin embedded tissues. When the latter material is cut, the knife is set at an oblique angle so that as

much of its cutting edge as possible is utilized. Its long axis, in other words, forms an angle of 45 degrees or more with the direction of its cutting motion.

The Freezing Microtome.—Whenever for some reason tissues are not embedded in either paraffin or celloidin, they may be cut in sufficiently thin sections with the freezing microtome (Fig. 371). The tissue, never more than 1 cm. in diameter, is placed upon the freezing chamber and frozen by means of ether spray or liquid carbon dioxide. The latter is the quickest and most satisfactory agent. It is available in large tanks which can be conveniently attached to this special type of microtome. Freezing chambers may be attached to the sliding microtome and used successfully. The Spencer Freezing microtome is an excellent instrument though the author prefers the German made Sartorius microtome, the knife of
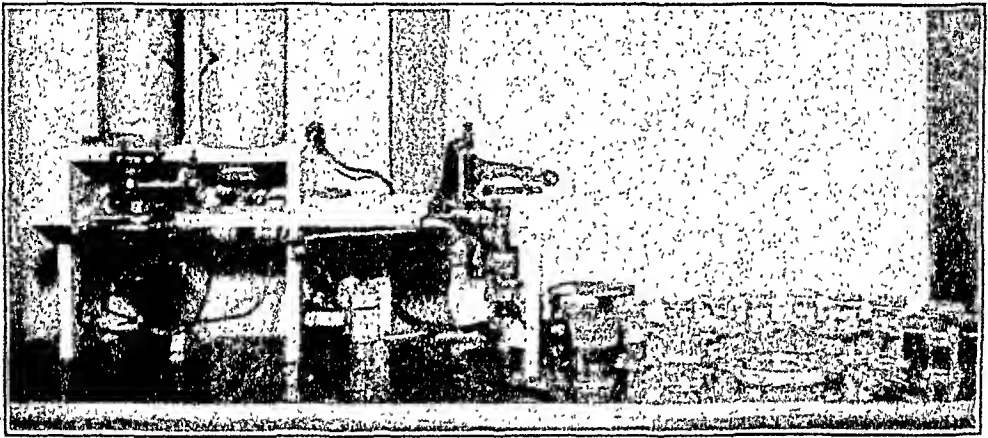
Fig. 371.—The Freezing Microtome Arrangement at Temple University Hospital Showing the H-tube so that Each Microtome May Receive CO_2 from Either Cylinder

which is supported at both ends in a far more stable manner. The same care must be given these microtomes as prescribed for the rotary type. It is to be emphasized that good sections cannot be obtained with a worn out microtome.

Microtome Knives.—These are the Waterloo of many a technician. All other requirements for good technic may be met, yet a poor knife will bring about complete failure. Good steel is the first essential. Knives sold by the leading optical companies seem to be of the finest steel. Those made by Spencer in the past have been too soft. They are easily sharpened but do not retain their edge. Knives imported from Germany in the years before the World War were too hard. It was extremely difficult to obtain a good cutting edge and nicks occurred more frequently than with the softer steel. Spencer's recently made knives and those of Bausch and Lomb or Arthur H. Thomas in Philadelphia seem to be of a desirable degree of hardness.

The practice of sending knives out to a grinder to be sharpened is not recommended unless it be for the removal of deep nicks or to "true" a knife that has been ground irregularly. The knife should be frequently honed and stropped if its edge is to be maintained. The microtome knife sharpener of

Schmidt or Fanz may be used successfully. One or the other should be in every laboratory and its care delegated to one individual. The directions for the use of either are fully given by the dealer who supplies them. The author has for years used the smaller hones and while good results are obtained, expertness is necessary. A honing guide must be fitted to the back of each knife and so marked that it is always placed upon the knife in the same manner. A medium coarse yellow Belgian hone is used for rapid grinding out of small nicks. The stone must be constantly flooded with water lest the particles of steel removed roll under the edge and produce additional nicks. Final honing is performed on a fine blue-green stone. The surface of this stone must likewise be flooded with water. A small accessory stone is provided with each hone for the purpose of keeping the surface flat and free of scratches. It should be rubbed upon the hone evenly (20 or 30 strokes) each time before the hone is used. The knife should be placed flat upon the distant end of the hone and drawn towards the worker cutting edge foremost, without pressure and bringing the whole cutting edge of the knife across the hone. As the mechanic expresses it, the knife is drawn from heel to toe (point). The knife is turned on its back (never the edge) and the opposite side is honed in the same way. These two motions are repeated until the desired edge is obtained (Fig. 372).

The first motion is as above described from heel to toe then, turning the knife on its opposite side, it is ground from toe to heel. It is turned again and ground this time toe to heel and the fourth motion after turning it from heel to toe. Thus each side of the edge is ground with a criss-cross motion. This serves to prevent a belly in the cutting edge and seems to increase the keenness of the edge. It is well to study the effect of the honing by frequent examination of the edge under the microscope using the 16 mm. objective. A good edge must be quite even and free of nicks. The scratches formed by the rough hone must be polished out by the fine hone and the strop.

Stropping.—Grinding or honing results in the formation of serrations like the teeth of a saw, in the cutting edge. These are extremely fine when the knife is properly honed. The purpose of the strop is to bring these serrations into a straight line. The flexible barber's strop of the best grade is most desirable although the flat mounted strops seem more popular. Strop dressing is not needed unless the principle of the barber be followed in occasionally rubbing in a quantity of shaving soap. This serves to keep the leather soft and pliable. Gouges and nicks in a strop are an evidence of inexcusable carelessness and indifference. Occasional stropping will keep a good knife in excellent condition for the cutting of many paraffin blocks.

A razor is a necessary instrument for trimming pieces of tissue. The old-fashioned straight type is the best. Its edge should be constantly maintained. The practice of using razor blades may be convenient but they are a needless expense. Many times a longer sturdier blade is needed.

The Paraffin Oven.—Any type which will maintain a constant temperature will prove satisfactory. The electrically heated ovens are the safest, for open

flames are a source of danger whenever such fluids as xylol, benzol or acetone are being employed. Dioxane, a dehydrating agent recently recommended, especially demands an electrically heated oven. Those fitted with a paraffin well have never proved satisfactory for the spigots have always leaked. The paraffin used for blocking may be kept in a beaker or enamel pitcher on a shallow tray in the oven.

Glassware.—The Coplin type of staining jar is the most satisfactory. Large Petri dishes of about 6 inches diameter and three inch depth may be used when
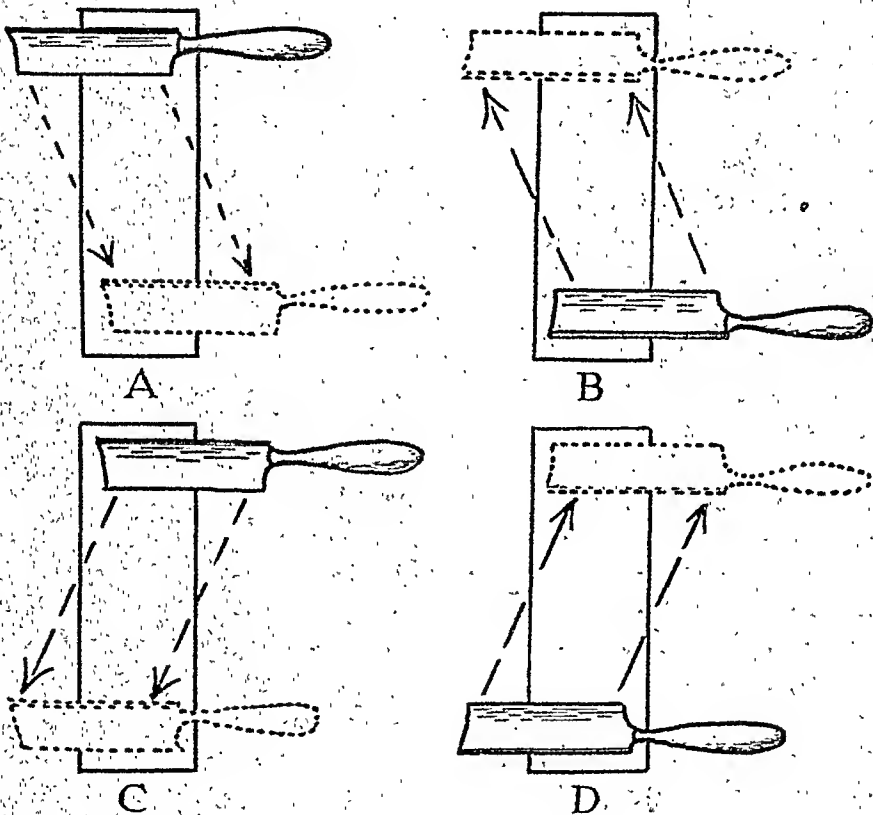
FIG. 372.—METHOD FOR STROPPING MICROTOME KNIVES

filled with warm water to receive paraffin section after cutting. The smaller varieties are suitable for staining celloidin sections. In place of the former a large pyrex baking dish, its bottom painted black, mounted in a suitably sized box, serves to receive paraffin sections. The ordinary 75 watt electric bulb mounted in the box will warm the water in the dish sufficiently to flatten out paraffin sections. Small stender dishes are convenient for staining frozen sections. Their glass covers prevent the evaporation of stains.

RAPID METHODS FOR PREPARING SECTIONS OF TISSUE

Ultropak Method.—The most rapid method of preparing tissue for microscopic study is that using the Ultropak microscope. This instrument utilizes an

objective built in an illuminator so that the light is reflected from above down upon the tissue on the microscope stage. The tissue, which should be fresh, is prepared by cutting a thick slice (1-3 mm.) with a sharp straight razor so that a plane even surface is obtained. The freshly cut surface is dipped in a thin layer of 0.3% solution of toluidine blue (Grübler) for 20-30 seconds and quickly washed with acidulated water. It is immediately placed upon the stage and examined. The tissue must be kept moist. The polychrome effect renders sharp differentiation between cells and stroma. After some experience one can diagnose malignancy as accurately as by the more tedious frozen method. Further, its permits the examination of larger areas.

Terry's Method.—The procedure just described has the disadvantage of requiring an expensive objective. Terry has devised a method wherein the ordinary microscope may be used. A thinner slice (1 to 2 mm. thick) is cut, with as before, a plane even surface. It is placed upon a microscope slide and Grübler's or Terry's modified polychrome methylene blue is painted on the exposed surface of the tissue. Staining must be done quickly (about 30 seconds) and care must be taken that no stain runs between the tissue and the glass slide. The tissue is flooded with water, a coverglass is placed over it and it is now ready for study. Here, only the superficial layer of cells is stained. The section is illuminated by the condenser of the microscope in the usual way. It is thin enough so that light passes through the unstained tissue readily. Considerable nuclear detail may be seen in these sections. The method is as adaptable as the previous one and reveals finer detail. With Terry's original method it is quite difficult to cut a thin slice possessing a plane surface. Unfixed tissue is apt to be particularly soft and difficult to cut. If unevenness of surface occurs a clear image cannot be obtained. Konzelmann uses a hemostat or a specially designed handle in which two razor blades are supported. The long Durham Duplex blade serves best. With the hemostat, the blades are separated by a narrow strip of cardboard of a thickness equal to that desired of the section (1 to 2 mm.). The cardboard should be as long as the blade and about 3/16 of an inch wide. It is placed along one edge between the two blades and then the whole grasped and locked firmly in the jaws of the hemostat. Thus a double-edged knife is formed. It is drawn downward into the tissue with an even sweeping motion. When it has entered the tissue to its full depth, the blades are tilted almost at a right angle to their former position and by a slight sawing motion the thin slice between the blades is severed from its point of attachment. The blades may then be separated and the section transferred to the slide. This procedure may be carried out routinely while another technician is preparing the frozen section. Often a diagnosis can be made before the tissue is completely frozen.

Frozen Sections.—If the sections are to be cut quite thin (1/100 of a millimeter—10 microns, or less) they must be rendered firm. In the slower methods this is brought about by infiltration with paraffin or celloidin. The tissue is altered by the solutions through which it is passed so that it neither looks the same microscopically nor is it fit for certain special stains. Fat, of course, would

be dissolved by the dehydrating fluids or clearing agents. Hardening the tissue by freezing is a method to be chosen where immediate sections are desired or whenever the solutions used in the slower methods render the tissue unfit for certain special studies.

Various freezing agents have been used but liquid carbon dioxide is probably the most efficient and convenient. Recently, carbon dioxide snow has been suggested by Lindsay and his colleagues and probably is just as serviceable. For the latter method a special block had been designed (Fig. 373) which may be used in a simple microtome to hold the tissue in contact with the "dry ice." The method is well worth considering because of its convenience. The liquid carbon dioxide method is the method of choice. The freezing agent is supplied in iron tanks or cylinders; several should be kept on hand for there is no way of telling when the one in use is nearly empty. It ceases to function suddenly. It is a wise custom to have two separate microtomes connected to separate cylinders or to connect two tanks to a single microtome. If one becomes exhausted, its valve may be closed and the second tank opened with negligible interruption. The cylinder should be mounted valve end down at an angle of 45 degrees or better still, on its end. It is the liquid carbon dioxide. in its transformation to a gas, that brings about the freezing. If only the gas is released as would occur when the cylinder is mounted valve up, there would not be sufficient heat extraction to freeze the tissue.
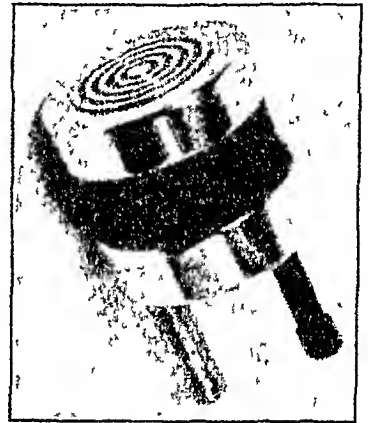
FIG. 373.—THE "DRY-ICE" CHAMBER OF LINDSAY *et al.*

The tissue may be either fresh, unfixed, or fixed in hot (60° C.) formalin for 3 to 4 minutes. Fresh tissue is frequently difficult to cut and to handle after it is cut but some stains will be effective only with fresh tissue. Cold formalin requires several hours to penetrate so if immediate sections are demanded, hot formalin must be used. It does cause shrinkage but the alteration is not great enough to defeat the purposes of this rapid method. The block of tissue should not be more than 3 mm. thick, nor more than 1 cm. on a side.

A little water is placed upon the freezing chamber. enough to form a pool about 2 mm. deep; the valve of the microtome is opened for a few seconds. The liquid thus released is immediately vaporized in the freezing chamber from which it issues in white clouds. Alternate releasing of the valve with intermissions of 5 or 10 seconds, conserves the freezing agent and is more effective than prolonged release. After the pool is *almost* completely frozen, the thin layer of unfrozen surface water should be wiped off with the finger. A flat instead of rounded surface is obtained to receive the tissue. This thin layer of ice protects the knife lest it strike the metal platform after the tissue has been cut entirely through. Orient the tissue squarely so that the first contact with the knife will be with one of the corners of the block. If the knife strikes parallel to one

of the sides of the block, the tissue will in all likelihood be dislodged. The tissue must be placed down in its proper position for as soon as it strikes the ice it will adhere and cannot be moved to a new position. After orientation cover the tissue with a little water and release the valve spasmodically until the tissue is frozen solid. If its surface is not now quite plane, it may be trimmed with a sharp knife or razor kept for that purpose. This conserves the edge of the microtome knife. Adjust the automatic feed to 10 microns.

Be certain that the surface of the tissue is 1 or 2 mm. below the edge of the knife and then with the right hand moving the knife in its sweeping stroke and the left advancing the micrometer screw one quarter of a turn before each stroke, the tissue gradually comes into the plane of the knife edge. As soon as it begins to cut, permit the automatic feed alone to raise the block. Continue until the knife cuts the entire surface. The sections until now will in all likelihood be shredded or powdery because the tissue is too cold. It may be made warm by applying the finger for an instant and then cutting again. When the correct degree of hardness is brought about by the gradual warming of the tissue, the sections will cut easily and curl up slightly upon the knife, from which they may be lifted with a camel's hair brush. If the knife is brought over the tissue with a swift even stroke that terminates suddenly but not too firmly as the knife carriage strikes the post which limits the movement, the section will be thrown from the knife. It may be caught in a dish of water which is held by the left hand to receive it. By either method the section is received in water or in 1% salt solution and may then be carried through any of the staining technies. A number of sections may be cut in a short time and there will be plenty of tissue available for staining. Usually it is not necessary to freeze again but if the tissue becomes too soft it may be done as at first. Sometimes when the tissue is lacy and breaks easily it is better to permit it to become softer and to allow the sections to pile up on the knife edge. After 8 or 10 have thus accumulated the whole mass can be wiped off with the finger and transferred to the dish of water. They will then slowly separate and can be unfolded by gentle poking with a small brush. The separate sections may now be transferred to a staining dish with a section lifter or a camel's hair brush.

If the tissue is very fragile it is well to wash out the fixative and infiltrate before freezing with some mucilaginous medium made as follows:

> Saturated aqueous solution of cane sugar............ 3 parts
> Gum acacia mucilage........................... 5 parts

Gum acacia mucilage is made by dissolving 60 grams of gum acacia in 80 c.c. of distilled water.

The tissue is placed upon the platform in a pool of this mucilage as described for water above and frozen. The gum solution should be removed from the sections by washing in water before staining.

For rapid staining the following method may be employed:

Transfer the sections to a small dish containing Unna's polychrome methylene

blue (Grübler) or Terry's modified polychrome methylene blue. The author has obtained good results with old solutions of Löffler's methylene blue. Keep the sections moving constantly for 10 to 20 seconds and then wash in 1% sodium chloride solution.

Transfer to a slide and mount in glycerin jelly or Brunn's glucose medium, made as follows:

Glucose.....................................	240 grams
Distilled water	840 c.c.
Spirit of camphor	60 c.c.
Glycerin	60 c.c.
Filter	

Dissolve the glucose in the water with gentle heating.

These sections are not permanent. Permanent sections may be prepared almost as rapidly. The section may be stained in dishes as above, using a small stender dish or watch glass for each solution or the section may be stained upon the slide. After transferring to a slide, drain the excess water and dehydrate with 80% and then absolute alcohol. Permit a few drops of a mixture of equal parts of ether and absolute alcohol to flow over the section and then cover with one drop of very thin celloidin (0.05%). Thin celloidin for this purpose may be prepared as follows:

1. Stock solution: One gram of celloidin in 50 c.c. alcohol and ether mixture.
2. Add 5 c.c. of this stock solution to 50 c.c. of alcohol and ether mixture.

A thin film fastens the tissue to the slide but does not interfere with staining. The section may now be stained in hematoxylin and eosin as are paraffin sections. They may be studied while still in creosote if protected by a cover glass or they may be at once permanently mounted in balsam.

METHODS FOR THE FIXATION OF TISSUES

Fixation is the process whereby tissues are prevented from undergoing any disintegrative or digestive change. There is no ideal fixing agent. Many cause shrinkage of the tissue.

Zenker's Fluid.—Potassium bichromate .... 2.5 grams
Mercuric chloride .... 8 grams
Water .... enough to make 100 c.c.
Add 5% glacial acetic acid before using.

The mercuric chloride will not all dissolve. The amount indicated will maintain a saturated solution which is required. Thin slices of tissue (not over 5 mm.) are fixed in 10 times their volume of the reagent for 12 to 24 hours. It is well to place a thin layer of cotton or paper on the bottom of the vessel so that the tissue does not stick to the glass. After fixation, wash the tissue for 24 hours in running water.

This reagent preserves well the structure of the tissue. Fibrin, fibrils and

nuclear details are sharply defined when this fixative is followed by phospho-tunstic acid hematoxylin. Tissues fixed in Zenker's fluid require longer hema-toxylin staining time than those fixed in other reagents.

Helly's Fluid.—This is similar to Zenker's; 5% formalin is added instead of glacial acetic acid. It preserves some types of cytoplasmic granules which are dissolved by acetic acid. It has the same advantages as Zenker's fluid and it is used in the same manner.

Flemming's Solution.—Osmic acid (2% aqueous solution) .... 4 c.c.
                 Chromic acid (1% aqueous solution) .. 15 c.c.
                 Glacial acetic acid .... 1 c.c.

It is best to make this solution fresh from the aqueous solutions just as it is needed. It penetrates slowly and therefore tissues should be not more than 2 mm. thick. Complete fixation requires 1 to 2 days. The tissue must be washed for 12 to 24 hours before dehydration. This is an excellent fluid for the preservation of fat in tissue. The fat is blackened by the osmic acid.

Alcohol.—This reagent in 95% or better, 100% concentration is a good fixative but it causes considerable shrinkage unless it is used in ascending concentrations starting with 70%. It hardens the tissue considerably. It is seldom used except where glycogen is to be demonstrated. Glycogen is very soluble in water and requires that the tissue be fixed in absolute alcohol and embedded in celloidin.

Formaldehyde.—Formaldehyde is a gas, soluble in water. It is obtainable in 40% solution commonly known as formalin or formol. It is used in 4% strength of the gas (10% of the commercial solution). Formic acid is generated in formalin solutions and has a deleterious effect upon tissue. Therefore, stock bottles of the prepared fixative should contain a thick layer of marble chips on the bottom for the purpose of neutralizing it. Formalin penetrates rapidly and fixes well. It hardens the tissue. It permits the use of a wide variety of stains. It is the best fixative for nerve tissue. It does not prevent shrinkage of tissue by alcohol in dehydration or by the heat of paraffin oven as well as Helly's or Zenker's fluids.

Formol-Alcohol.—A mixture of these two reagents has the advantage of fixing and dehydrating at the same time. There is considerable shrinkage of tissue which, however, does not greatly interfere with routine studies. It is frequently used for rapid paraffin or celloidin methods. The formula used at the Temple University Hospital is as follows:

Formalin (40% formaldehyde solution) .... 18 c.c.
Alcohol (95%) .... 60 c.c.
Glacial acetic acid .... 3 c.c.
Water .... 39 c.c.

Pieces of tissue from 2 to 4 mm. thick are fixed in 1 to 4 hours.

Picro-Formol-Acetic Acid Mixture.—These mixtures are well known because of the excellent manner in which they preserve cytologic details. Some nuclear

aniline dyes do not stain well after fixation in this fluid. If Bouin's fluid or a modification is used some tissues should be fixed in Helly's or Flemming's fluid.

Bouin's Fluid.—Saturated aqueous solution of picric acid.... 75 parts
Formalin (40%) .... 25 parts
Glacial acetic acid .... 5 parts

There is little danger of overfixation and the fluid penetrates rapidly.

McClung and others have found that the addition of urea enhanced the preservation of finest cellular details. Allen's modification is recommended:

Saturated aqueous solution of picric acid.......... 75 parts
Formalin (40%) C.P. .... 15 parts
Glacial acetic acid .... 10 parts
Urea .... 1 part

The addition of chromic acid, 1 part, to Allen's formula is also recommended. It must be added just before using for the chromic acid is reduced by the formol with the development of a greenish color. Other discolorations or precipitates are due to impurities in the reagents used and render the fluid useless.

WASHING

Tissue need not be washed in water after formalin or alcohol fixation. Tissues fixed in solutions containing chromium salts should be washed for 24 hours in running water. Those fixed in picric acid solution will be macerated if left too long in water; washing should therefore be brief (several hours).

METHODS FOR THE DEHYDRATION OF TISSUES

Ethyl Alcohol.—This is the most common dehydrating agent. It should be used in ascending strength, 70%. 80%. 95%. and absolute alcohol. Where one wishes to reduce shrinkage to a minimum, still weaker solutions must be employed. Regardless of the manner of dehydration with alcohol, there will be shrinkage but it will be less with the slower and more gradual process. Iso-butyl alcohol has been used with slightly less shrinkage than that caused by ethyl alcohol. It is toxic and its vapors may cause headache.

Dioxane (Diethyl Oxide).—This is a colorless liquid which boils at 101° C. It is a solid below 8° C. It is highly volatile. mixes with all proportions of water and alcohol. It dissolves paraffin slightly when cold and quite readily when heated. According to Baird and Bucher while it does not completely meet the requirements of a perfect dehydrating agent it is definitely superior to any substance used at present. Tissues remain soft and do not become brittle. There is little or no shrinkage depending upon the tissue itself. and there is no distortion. In Bucher's and Blakely's experience. dioxane has enhanced rather than detracted from the effect of some of the commonly used stains. No special preparation of the tissue is necessary except for colloid or colloid-like substances which have been fixed in formalin. These must be washed in running water for 12 or 24 hours.

Under other circumstances, tissues are treated as for dehydration by alcohol. It is most important to remove all paraffin from the section before staining, for even traces of paraffin will interfere. Dioxane is inflammable and no attempt should be made to redistill it. There must be no open flame in the room where it is employed. Its vapors have a slight anesthetic effect. Therefore, all containers must be kept covered or tightly stoppered.

Bucher and Blakely have designed a small perforated cup of porcelain which rests in a stender dish (93 mm. in height and 62 mm. in diameter) upon glass supports so that the tissue in the cup lies at about the middle of the fluid volume.[1] Dishes are filled three-fourths with the reagent. Their technic is as follows:

1. Fixed tissue not more than 4 mm. thick is placed in a mixture of 1 part distilled water and three parts by volume of dioxane (unrefined, commercial). This step lessens shrinkage and may be omitted. One hour is sufficient time in this mixture.

2. The second dish contains pure dioxane. Anhydrous calcium chloride is placed in the bottom of the dish to about the depth of 1 cm. It prolongs the usefulness of the reagent for after about 2 weeks it may be removed, the dioxane filtered and used again. Two to three hours is sufficient to complete dehydration. A longer time does not harm the tissue.

3. Infiltration after dioxane does not differ greatly from the procedure commonly followed. The tissue is supported above the bottom of the paraffin container by copper wire baskets (about the size and shape of the porcelain cups). As the dioxane is volatile, several changes of paraffin are not necessary. The tissue has a tendency to fall out of ribbons if all the dioxane is not removed. Infiltration for a period of 8 to 12 hours seems to accomplish complete removal and thorough infiltration. We recommend paraffin having a melting point of 56° to 58° C.

The method of staining is that commonly used. Alcohol instead of dioxane is employed as a dehydrating agent for sections. The authors mentioned above prefer Harris's hematoxylin stain followed by immersion in 2% aqueous solution of sodium hyposulphite for 15 to 30 seconds. The section is of course to be washed in water after each step. Xylol is used as a clearing agent before mounting in balsam.

Acetone.—While acetone is suitable for small fragments of tissue it is not generally recommended for it produces shrinkage and shattering of tissue. Small fragments may be dehydrated by 2 or 3 changes of acetone of ½ to 1 hour each.

METHODS FOR THE CLEARING OF TISSUES

Since ethyl alcohol is not miscible with paraffin or celloidin, the embedding substances to be employed, it is necessary to find some substance which will remove the alcohol from the tissue and be in turn miscible with paraffin. Iso-butyl alcohol or dioxane require no clearing agents for these substances will mix with paraffin. There are three commonly used clearing agents. Some, but not all of these, render the tissue translucent, hence the name of the procedure is inappropriate.

[1] May be purchased from A. H. Thomas Co., Philadelphia.

Cedar-Wood Oil.—Tissues should be placed after dehydration in a mixture of equal parts of cedar-wood oil and absolute alcohol and then in pure cedar-wood oil. From 2 to 12 hours are required for each depending upon the nature of the tissue. The tissue is translucent when completely "cleared." The oil should be washed from it with chloroform for several minutes before placing it in paraffin. Several changes of paraffin are required to remove the last traces of oil. Some workers prefer to place the tissue in a mixture of equal parts of oil and paraffin and then into two changes of paraffin.

Benzol.—Benzol may be used for small pieces of tissue that clear quickly. It hardens too much when treatment is prolonged. One-half to one hour is required for fragments 2 to 3 mm. in diameter. When the action is complete the tissue is quite translucent.

Chloroform.—Tissues are placed in pure chloroform after dehydration. After 4 to 8 hours they may be transferred to a mixture of equal parts of chloroform and paraffin where they remain for 4 to 8 hours.

PARAFFIN METHODS FOR THE INFILTRATION AND EMBEDDING OF TISSUES

Infiltration.—Infiltration is designed to give the tissue rigidity so that it may be cut in extremely thin sections, and to hold together its fragments. Paraffin and celloidin are commonly employed. Paraffin is the most popular infiltrating agent. That having a melting point of 56° to 58° C. is chosen. In very cold weather, that having a melting point of 52° C. is more desirable. It is recommended that a large supply be kept on hand, for new paraffin tends to crystallize, a change which renders section cutting impossible. Infiltration is obviously carried on in some form of oven that maintains a constant temperature slightly above the melting point of the grade used (1 to 2 degrees). It should never be permitted to go above this point lest the tissues be damaged. Mixtures of paraffin and cedar-wood oil or chloroform should be placed on top of but not in the oven. Some prefer two changes of paraffin. The first having a melting point slightly lower than the second or final bath. Tissues of the thickness recommended (3-5 mm.) are usually completely infiltrated at the end of 4 or 6 hours. However, they are frequently left in the paraffin oven over night. Some workers have found the addition of rubber improves the quality of the sections obtained. The following formula is recommended (Baird):

Prepare a saturated solution of crude rubber in paraffin (m.p. 53°-55° C.)

This is the stock rubber-paraffin, with which the embedding medium is prepared.

Stock rubber paraffin (winter months)	160 grams
(summer months)	140 grams
Paraffin (m.p. 53° to 55° C.)	720 grams
Bayberry wax	80 grams
Aniline oil	5 c.c.

It may be used as plain paraffin.

Embedding.—Tissues may be embedded in paraffin on wooden blocks, in paper cups or in Petri dishes. Oak blocks 1 x 1 x 1.5 inches are the most suitable. Wrap a piece of paper 2½ x 6 inches, around the block so that a cup is formed on one end (Fig. 374). A rubber band will hold the paper in place. Pour some hot melted paraffin into the cup and set the tissue in it keeping in mind the manner in which it is desired that the tissue appear in the sections. For example a piece of intestine must be placed on its edge so that the microtome knife will cut cross sections of its wall. Fill the cup with paraffin until the tissue is well covered. Set the block aside

1. Crease on dotted lines

2 Fold up one end and one side. Fold projecting flap against the end.

3. Repeat with adjoining corner

4. Fold over and crease the projecting end flap to lock the corner joints.

Fig. 374.—The Method of Folding the Paper Cups
(From Gradwohl, *Clinical Laboratory Methods and Diagnosis*, C. V. Mosby Co.)

until the surface solidifies. Then plunge the entire block into ice water or stand it next to ice in the refrigerator until it has completely hardened. If the metal disk is used to carry the paraffin block in the microtome, embedding must be done in paper cups or in a Petri dish. The block prepared by any method must be neatly trimmed and the edges beveled so that the surface is a true rectangle. The sections will separate more readily if the corners of the block are cut away. The tissue should be well surrounded by paraffin on all sides. Chill the block on ice before placing it in the microtome. Routine sections should be cut 6-8 microns thick. If the tissue has been properly infiltrated it will form long ribbons as sections are cut. Transfer the ribbon to a dish of warm water (40° to 45° C.) with the aid of a camel's hair brush and fine forceps. If the water is of the correct temperature

(40° to 45° C.), the sections will flatten out completely and they may be separated by poking gently at the nick that has formed between the sections as the result of cutting the corners of the block. Float a section upon a slide which has been smeared with Mayer's albumin:

This adhesive material may be prepared by mixing equal parts of egg white and glycerin. Beat the mixture thoroughly until it flows evenly like thin syrup. Filter and add 1% sodium salicylate as a preservative.

Place a tiny drop upon a slide and smear evenly over the entire surface with the finger. The excess may be wiped away with palm of the hand. Only a very thin film is desired. After the sections have been properly located, permit them to dry well protected from dust for several hours or better overnight.

SUMMARY OF METHODS FOR INFILTRATING AND EMBEDDING IN PARAFFIN

Slow Method.—1. Fixation of tissue not more than 5 mm. thick.

  10% formalin ....12-24 hours or,
  Zenker's fluid ....12 to 24 hours or,
  Bouin's fluid ....6 to 12 hours
  Wash in running water....12 to 24 hours after Zenker's.

2. Dehydration.

  Ethyl alcohol 70% .....12 hours
  Ethyl alcohol 80% .....12 hours
  Ethyl alcohol 95% .....12 hours
  Ethyl alcohol 100% .....12 hours

3. Clearing.

  Ethyl alcohol 100% and
  Cedar-wood oil equal parts 12 hours
  Pure cedar wood oil ......12 hours

4. Infiltration.

  Cedar-wood oil and
  Paraffin equal parts ......12 hours (Place on top of oven)
  Paraffin 1 .... 8 hours
  Paraffin 2 .... 8 hours

5. Orientation and embedding.

  Cool block quickly.

Rapid Method.—Tissue must not be more than 3 mm. thick.

1. Fix in formol-alcohol 1-4 hours.
2. Dehydrate in acetone, 3 changes each ½ hour.
3. Clear in benzol for ½ hour or until translucent.
4. Infiltrate with paraffin for 2-4 hours or overnight.
5. Orient and embed as in method No. 1.

Dioxane Method (Bucher and Blakely).—1. Fixation as in method No. 1

2. Dehydrate in dioxane mixture for 1 hour:

  (a) Dioxane ....2 parts
    Water ....1 part

(b) Pure dioxane for 2-3 hours.
3. Infiltrate with paraffin 8-12 hours.
4. Orient, embed as in method No. 1.

CELLOIDIN METHODS FOR THE INFILTRATION AND EMBEDDING OF TISSUES

It was once believed that paraffin could only be used for small sections of tissue, but Woinwright and others have succeeded in preparing beautiful sections of whole breasts and other organs embedded in paraffin. There are tissues, however, which are damaged or hardened by the heat of the paraffin oven so that some embedding material is desired that may be employed at room temperature. This requirement is met by trinitrocellulose, sold for this purpose under the name of Celloidin or Parloidin. Some workers have found commercial guncotton as sold by the Hercules Powder Co. to be just as satisfactory and considerably cheaper. It may be purchased in 5 pound tins and of course must be carefully stored, far removed from any source of heat. The cans must never be opened with a steel instrument lest a spark be generated and the guncotton ignited. Celloidin is used for embedding in two or more solutions. The first is thin, just half the strength of the stock or thick celloidin. Thick celloidin is prepared by dissolving 30 grams of dried material in 200 c.c. of a mixture of equal parts of absolute alcohol and ether (Mallory). One must carefully dry the chips of celloidin, since they are preserved in water. Parloidin is now obtainable in dry glass bottles. It is customary to place the wet chips upon a piece of absorbent paper for 24 hours far removed from any heat source or flame. They should be protected from dust. While large pieces of tissue such as whole brain sections may be embedded in celloidin, tissues cut better if they measure not more than 2 cms. on a side and 5 mm. thick. The trimmed pieces are fixed and dehydrated in the usual manner. After dehydration in absolute alcohol, they are transferred to a mixture of absolute alcohol and ether where they remain for 24 hours. They may then be transferred to thin celloidin. Here they must remain for at least 24 hours or better, for several days. Finally the pieces are soaked in the thick solution for one or more days. The pieces should then be grasped with a pair of forceps carrying as much of the celloidin with them as possible and oriented on a vulcanized fiber block. Block and tissue are immediately plunged into chloroform where they are held submerged for 1 or 2 hours. After this they may be stored in 80% alcohol. Some prefer to place the tissue in moderately thick celloidin in a dish fitted with a tight cover. After sufficient soaking one or more days, the cover is lifted for a few hours permitting a film of celloidin to form on the surface. The lid is replaced and overnight the vapor of ether dissolves the surface film with consequent concentration of the mass of celloidin including that which has infiltrated the tissue. This process is repeated until the mixture acquires a moderately firm consistency. Blocks may now be cut out with a knife, cemented to the fiber blocks with thick celloidin, plunged into chloroform as before and finally stored in 80% alcohol. Sections may be cut on any microtome though the sliding type is preferred. The knife must be set at an angle of 45 degrees or more

so that nearly all the cutting edge passes through the tissue. The block and the knife must be kept wet with 80% alcohol. The sections may be transferred to water or 80% alcohol where they remain until stained. They may be stained upon the slide or in staining dishes. Nearly all methods are applicable without removal of the celloidin. If it becomes necessary, oil of cloves or the alcohol and ether mixture may be employed to dissolve the celloidin. If celloidin sections are carefully blotted and the wrinkles ironed out, they will ordinarily adhere quite firmly to the slide. They must never be permitted to become dry during the staining process.

Celloidin Method (Custer).—1. Fix tissue in formalin or Zenker's solution

2. Wash in running water for 24 hours

3. Dehydrate in alcohol

65% (with iodine for Zenker's fixed tissue)	12 hours
85%	12 hours
90%	24 hours
95%	24 hours
100%	24 hours
100% (with anhydrous copper sulphate as a desiccator).	24 hours
100% alcohol and ether, equal parts (with copper sulphate)	24 hours

4. Infiltrate. Pass through 5 solutions of celloidin from very thin to very thick, each 2 days.

5. Place in a covered dish containing thick celloidin and control evaporation so that the celloidin hardens from within outward until it has the consistency of hard rubber.

6. Cut cubes and preserve in 80% alcohol.

7. Dry cube and cement to fiber block with thick celloidin. Harden in 65% alcohol.

8. Keep knife and block wet with 65% alcohol while cutting. Very thick celloidin is made by dissolving about 22 grams of the chips in 100 c.c. of alcohol and ether mixture.

METHODS FOR THE STAINING OF TISSUES

The manner in which tissues or cells become stained when exposed to certain dyes is not at all clear. Once, it was supposed that the phenomenon was a chemical one, that basic substances would be stained by acid dyes and *vice versa*. At least it is not always so, for there is much to indicate that the process is purely physical, in which capillarity and osmosis account for the penetration of the dye. Adsorption may explain many of the phenomena of differential staining. Adsorption is a property possessed by a solid body, of attracting to itself, minute particles of matter from a surrounding fluid. These particles may exist as ions. The fact that the rate of adsorption may be influenced by the presence of other ions and that the reaction of the solution has a profound effect explains the phenomena of differential staining of various cellular elements, variation in the rate of staining with changes in the salt content of the staining solution and the

influence of the H-ion concentration upon color assumed by tissue when it is exposed to the action of both acid and basic dyes. Only the best available stains should be purchased for preparing solutions. For many years only the dyes of German manufacture could be relied upon and there are still some obtainable from Grübler that cannot be equaled. However, the dyes prepared by the National Aniline and Chemical Company, or Coleman and Bell in this country have given very satisfactory results. Whenever possible stains should be purchased which have been certified by the Commission on the Standardization of Biologic Stains of Geneva, New York.

Hematoxylin and eosin are the stains used routinely employed; they are prepared as follows:

Hematoxylin (Delafield):

> Hematoxylin crystals .... 4 grams
> Alcohol 95% .... 25 c.c.
> Saturated solution of ammonium alum... 400 c.c.

A saturated solution of alum should be kept on hand. It is made by dissolving 180 grams of ammonium alum in 1000 c.c of water by the aid of heat. When the solution cools, some of the alum will crystallize, but the supernatant fluid will be saturated. Dissolve the hematoxylin in the alcohol and add it to the alum solution. Expose the solution to sunlight and air in an unstoppered bottle for 3 or 4 days. Filter and add:

> Glycerin .... 100 c.c.
> Alcohol 95% .... 100 c.c.

Permit the solution to stand in the light until it is quite dark. It keeps well and its state of preservation may be determined by pouring a little into a beaker of water. A good stain develops a purple color while a stain that is poor or no longer effective causes a red solution. The action of hematoxylin depends on the presence of alum. Old solutions which stain too diffusely may be restored by the addition of alum to the stock. Precipitates are constantly forming; therefore, the solution must be filtered before it is diluted for use. It is the author's practice to dilute the stock with an equal volume of water before using.

Hematoxylin (Harris):

> Hematoxylin .... 1 gram
> Alcohol .... 10 c.c.
> Dissolve the hematoxylin in the alcohol.
> Alum (ammonium or potassium)....... 20 grams
> Distilled water .... 200 c.c.

The addition of 3 c.c. of glacial acetic acid increases nuclear staining.

The alum should be dissolved in the water by the aid of heat and then the alcoholic solution of hematoxylin added. Heat the mixture to the boiling point and add half a gram of mercuric oxide. As soon as the mixture develops a dark

purple color, cool it quickly by plunging the vessel which contains it into cold water. It is ready for use when it has cooled. The solution stains well and may be kept for a long time. It is especially recommended for Zenker fixed tissues.

Eosin.—Eosin Y (yellowish) is one of the most valuable of plasma stains. It is labeled as water soluble but it is also soluble in alcohol. It is made as follows:

> Eosin Y (85% dye content) .... 1.0 gm.
> Ethyl alcohol (95%) .... 25.0 c.c.
> Water .... 75.0 c.c.

Sections are stained from ½ to 1 minute.

Eosin B (bluish) is not a satisfactory stain. A bluish tint may be obtained by the addition of rose bengal or similar dye to eosin Y. Coleman and Bell offer such a product which is labeled "eosin bluish blend."

Paraffin sections are stained upon the slide. There are many types of staining jars available. The Coplin jar will accommodate 10 slides at a time for in it there are 5 grooves in each of which two slides may be placed back to back. Glass or metal racks carrying from 10 to 25 or more slides may be purchased when a great number of slides are to be stained at one time. The Coplin jar is the most popular; it is used for all of the reagents in the following steps:

1. Remove the paraffin by placing the slide in xylol for 5 minutes. A second bath in xylol is advocated for 3 minutes.

2. Wash in alcohol (95%) for 5 minutes to remove the xylol.

3. If the tissue has been fixed in Zenker's fluid, place the slide in dilute tincture of iodine for 5 minutes to remove the precipitated mercuric salts.

This step is necessary only after Zenker's fixation. Some prefer to remove the mercuric salts by dissolving iodine in the first alcohol in the process of dehydration. This solution must have enough iodine dissolved in it to give a port wine color. It must be renewed as frequently as the iodine fades.

4. Wash in alcohol for 5 minutes to remove the iodine.

5. De-alcoholize the tissue in descending strengths of alcohol (80% and 70%) each for 3 minutes.

6. Wash in water.

7. Stain in hematoxylin for 5 minutes.

8. Wash in water and place in acid bath (1% aqueous solution of hydrochloric acid) to remove the hematoxylin (½ minute) from all but the nuclei. Control decolorization by frequent examination of the tissue with the microscope. The stain must remain in the nuclei.

9. Wash in water. If one drop of ammonium hydrate is added to a jar full of water, the stain will deepen quickly. The ammonia must be thoroughly removed by washing before the next step.

10. Allow the slide to stand in pure water for 5 minutes to deepen stain.

11. Stain in eosin (1% aqueous, water soluble, yellowish eosin) for ½ to 1 minute.

12. Wash in water and then dehydrate in ascending strengths of alcohol—80%, 95% and absolute alcohol. Each solution for 3 minutes.

13. Clear in beechwood creosote or xylol for 5 minutes.

The section must be translucent. If xylol is used and tissue is not thoroughly dehydrated, a milky fluid will be formed which can be removed by returning the slide to the alcohol bath.

14. Drain and wipe the clearing fluid from the slide around the section. Place a drop of canada balsam on the tissue, cover with a cover glass. If the balsam does not immediately spread over the section without the inclusion of air bubbles under the cover, it is too cold or too thick. Gentle warming followed by gentle pressure will accomplish the desired result. If the balsam is quite thick, add a little xylol and mix until it has a thin syrupy consistency. It is important to add just the right sized drop of balsam. If too much is added, it will flow from beneath the cover and cause a sticky, dirty preparation. It is important also that not too much mounting media be used lest it interfere with the distinctness of the microscopic image, or prevent the use of the oil immersion lens. If too little balsam is used, the whole section will not be covered, air bubbles will form and the tissue will eventually dry out.

Mallory's Phloxine and Methylene Blue Stain.—This was formerly called the eosin methylene blue method until it was discovered that some of the staining qualities of this method were really due to the presence of phloxine in what was incorrectly sold as eosin. This mixture produces a sharp nuclear stain and reveals with marked differentiation the various structures in tissues. Tissues should be fixed in Zenker's fluid.

1. Stain paraffin sections in a 5% aqueous solution of phyloxine (dye content about 80%) for 20 minutes or longer.

2. Wash away the excess phloxine with water.

3. Stain for 30 minutes in borax methylene blue solution diluted in 10 parts of water:

Methylene blue (medicinal 90% dye content)..	1 gram
Borax	1 gram
Water	100 c.c.

Pour this solution on and off the section several times.

4. Wash in water.

5. Differentiate and dehydrate in 95% alcohol in a dish to which has been added a few drops of 10% collophonium. The section must be kept in constant motion so that decolorization will be uniform. Control the result under the microscope. When the pink color has returned to the section and the nuclei are still blue, complete dehydration quickly with absolute alcohol.

6. Clear in xylol.

7. Mount in balsam.

Phosphotungstic Acid Hematoxylin (Mallory).

Hematein ammonium	0.1 gram
Water	100.0 c.c.
Phosphotungstic acid crystals (Merck)..	2.0 grams

Dissolve the hematein in a little water with the aid of heat. After it is cool add it to the phosphotungstic acid dissolved in about 80 c.c. of water. Dilute finally to 100 c.c. The solution should be permitted to stand for several weeks to ripen before it is used. However, ripening may be hastened by the addition of 5 c.c. of a 0.25% solution of potassium permanganate. This permits immediate use of the stain. Hematoxylin may be substituted for the hematein ammonium. If this substitution is made, add 10 c.c. of the permanganate solution to ripen the mixture.

Zenker fixed tissue is required for this stain. Kernohan has obtained satisfactory results with formalin fixed tissue after it has been especially treated in Weigert's mordants.

1. Wash the tissue for several hours in running water or in dilute ammonia for a short time.

2. Fix four days in Weigert's primary mordant for myelin sheaths:

Potassium bichromate	5.0 grams
Chromium fluoride (flurochrom)	2.0 grams
Water	100.0 c.c.

3. Fix 2 days in Weigert's secondary mordant for myelin sheaths:

Copper acetate	5.0 grams
Chromium fluoride	2.5 grams
Acetic acid (36%)	5.0 c.c.
Water	100.0 c.c.
Formalin	10.0 c.c.

Nuclei. fibroglia. myoglia, neuroglia fibrils, fibrin and contractile elements of striated muscle are stained blue; collagen and ground substances of bone, varying shades of yellow or brownish red. Elastic fibrils develop a purplish tint. Tissues are run through the preliminary steps as for the routine staining method until they are brought into the first wash water after the removal of paraffin:

1. Place in 0.25% aqueous solution of potassium permanganate for 5-10 minutes.

2. Wash in water.

3. Oxalic acid, 5% aqueous solution for 10-20 minutes.

4. Wash thoroughly in water.

5. Stain in phosphotungstic-acid hematoxylin for 12-24 hours.

6. Transfer to 95% alcohol and then to absolute alcohol. Dehydrate quickly for the red stain is easily extracted.

7. Clear in xylol and mount in balsam.

Aniline Blue Collagen Stain (Mallory).—This stain is useful for the study of collagen, fibrin, fibroglia, muscle and amyloid. Collagen fibrils and reticulum of connective tissue, amyloid and mucus are stained blue; nuclei, cytoplasm, fibroglia fibrils, neuroglia fibrils, axis cylinders and fibrin red; red blood cells and myelin sheaths yellow; elastic fibers, pale pink or yellow. The dye is prepared as follows:

Aniline blue soluble in water (certified)	0.5 gram
Orange (certified) G. 80%-85% dye content	2.0 grams
Phosphotungstic acid	1.0 gram
Water	100.0 c.c.

Prepare tissues as for the routine stain to the first wash water.

1. Stain sections in 0.25% aqueous solution of acid fuchsin for 30 minutes. Drain and immediately:

2. Stain in the aniline blue solution for 1-24 minutes or longer. Staining in paraffin oven for 1 hour gives as good a result as over night in the cold.

3. Wash in several changes of 95% alcohol and then in absolute alcohol.

4. Clear in xylol.

5. Mount in balsam.

If the tissue is first stained lightly in Delafield's hematoxylin, nuclei and smooth muscles stain brownish color.

Azocarmine Modification (Haidenhain) of Mallory's Aniline Blue as Employed by McGregor for the Study of Renal Glomerulus.—In general the results are best following Zenker or Helly fixation. However, tissues fixed in 10% formalin may be treated as follows:

(a) For paraffin sections of formalin-fixed tissue:

1. Ammonia (40 drops to 10 c.c. water)	1 hour
2. Running water	1 hour
3. Zenker's or Helly's fluid	1 hour
4. Running water	1 hour

(b) For blocks of formalin-fixed tissue:

1. Ammonia (40 drops to 100 c.c. of water); 2 days in paraffin oven at 40° C.	
2. Running water	24 hours
3. Zenker's fluid	5 hours
or	
Helly's fluid	12 hours
4. Running water	12 hours

1. 1% azocarmine G (1 gm. in 100 c.c. water, heat, cool, filter at room temperature and add 1 c.c. glacial acetic acid). Stain 30 to 40 minutes in the paraffin oven at 57° C.

2. Wash in water,

3. Differentiate in aniline alcohol (1 c.c. aniline oil in 100 c.c. 95% alcohol). Watch under the microscope until the nuclei are red and the cytoplasm pale pink. This step requires from one to three minutes. depending on the thickness of the sections.

4. Remove aniline with acid alcohol, about one minute.

5. Three hours in 5% phosphotungstic acid.

6. Wash quickly in water.

7. Stain 3 to 6 hours in the following:

> Aniline blue .... 0.5 gram
> Orange G .... 2.0 grams
> Glacial acetic acid .... 8.0 c.c.
> Distilled water .... 100.0 c.c.

Boil. cool and filter. Dilute one-half with water.

8. Wash in water.

9. Differentiate in absolute alcohol, watching under the microscope.

10. Xylol.

11. Balsam.

Nuclei appear orange-red, cytoplasm pink, connective tissue and reticulum blue, fibrin red.

Van Gieson's Stain.—This is a differential stain for collagen which assumes a red color. All other tissues are stained yellow. Best results are obtained with material fixed in chromium salts (Zenker's fluid). The stain is prepared as follows:.

> 1% aqueous solution of acid fuchsin..... 5 c.c.
> Saturated aqueous solution of picric acid.. 100 c.c.

Solutions must always be tested upon tissues containing collagen. If the collagen does not stain a definite red a little more of the acid fuchsin must be added. The solution may lose its differential staining qualities if exposed to the light for a long period. Therefore it should be tested from time to time. Sections are stained in the following manner:

1. Stain deeply with alum hematoxylin as in the routine method.

2. Wash in water and stain with Van Gieson's mixture 3-5 minutes.

3. Dehydrate in 95% alcohol.

4. Clear in oil of origanum (pure).

5. Mount in balsam.

Unna's Alkaline Methylene Blue Solution.—This solution was recommended by Unna for staining plasma cells:

> Methylene blue (90% dye content)..... 1 gram
> Potassium carbonate (Merck) .... 1 gram
> Water .... 100 c.c.

Its chief value is in the preparation of Unna's polychrome methylene blue. The alkaline solution is permitted to ripen for months when, as the result of oxidation,

methyl violet and methyl-red are formed. The fully ripened solution may be obtained from Grübler. Dilute 1-10 before staining.

Terry's Neutralized Polychrome Methylene Blue.—This is a modification of Goodpasture's formula. Three aqueous stock solutions are prepared:

1. 12% anhydrous potassium carbonate.
2. 1.0% methylene blue (medicinal—90% dye content).
3. 10% (by volume) acetic acid.

One c.c. of solution 3 is titrated with solution 1 using phenolphthalein as the indicator. The amount of solution 1 required to exactly neutralize 1 c.c. of solution 3 at the boiling temperature is introduced into a 100 c.c. cylinder and enough of solution 2 is added to bring the final volume to 100 c.c. This mixture is then divided into 4 equal parts of 25 c.c. Each part is placed in a one ounce bottle and four bottles in a basin of cold water which is gradually heated to boiling. The time of boiling is noted and at the end of 15 minutes one bottle is removed, the others at the end of 20, 25, and 30 minutes respectively. They are permitted to cool slowly and to each is added 0.25 c.c. of solution 3. The solutions are now ready for use though they will improve upon standing. Filtration is usually not necessary and should not be done for one or two days. If one determines by trial which of the four bottles contains the best stain, the next lot may be boiled for that length of time which gave the best results.

Verhoeff's Elastic Tissue Stain.—Formalin or Zenker's fixative may be used in preparation for this stain but the best results are obtained with the latter. Tissues are not to be treated with iodine for the staining solution itself will remove the precipitated mercuric salts. The solution must be freshly prepared for it does not keep longer than one month:

Hematoxylin crystals .... 1 gram
Absolute alcohol .... 20 c.c.

Dissolve by the aid of heat, filter and add in the order given:

Ferric chloride (10% aqueous solution) ..... 8 c.c.
Lugol's solution (iodine, 2 parts; potassium iodide, 4 parts; water, 100 parts) .... 8 c.c.

1. Stain sections until perfectly black (15 minutes).
2. Differentiate in 2% aqueous solution of ferric chloride. This step requires but a few seconds. Control it by examining the section with the microscope. If carried too far the section may be restained.
3. Wash in water and then in 95% alcohol to remove the iodine.
4. Wash in water again and stain for 1 minute with eosin.
5. Dehydrate, clear and mount as in routine method.

By this method, elastic tissue is stained black but connective tissue, fibroglia, myoglia, and neuroglia are stained red by the eosin.

Best's Carmine Stain for Glycogen.—Glycogen is quite soluble in water; therefore, when it is to be demonstrated, aqueous fixatives cannot be used. Abso-

lute alcohol is the best fixative and celloidin the best infiltrating agent. Of course the sections cannot be placed in water but may be placed in alcohol until stained. The staining mixture is prepared as follows:

Carmine .... 2 grams
Potassium carbonate .... 1 gram
Potassium chloride .... 5 grams
Water .... 60 c.c.

Boil gently for several minutes but do not overheat. Cool the solution and add:

Ammonium hydrate .... 20 c.c.

The mixture will keep for several months in tightly stoppered bottles. It should be filtered and diluted before using in the following manner:

Carmine solution .... 2 c.c.
Ammonium hydrate .... 3 c.c.
Methyl alcohol .... 3 c.c.

1. Stain sections deeply with hematoxylin.
2. Decolorize with acid alcohol if the stain is too diffuse.
3. Wash quickly but thoroughly in water.
4. Stain with the diluted carmine solution for 5 minutes.
5. Differentiate in the following mixture:

Absolute ethyl alcohol ....:. 80 c.c.
Methyl alcohol .... 40 c.c.
Water .... 100 c.c.

6. Wash in 80%; then in absolute alcohol.
7. Clear and mount in balsam.
By this method nuclei are stained blue and glycogen a bright red.

Amyloid Stain.—Fresh or formalin-fixed frozen sections are best suited for the demonstration of amyloid. Celloidin sections do not stain with the aniline dyes unless the celloidin is removed.
1. Stain frozen sections in 1% aqueous solution of methyl-violet for 5 minutes.
2. Wash in 1% aqueous solution of acetic acid.
3. Wash thoroughly in water to remove the acid.
4. Mount in glycerin jelly.
Amyloid is stained red, other tissues are blue violet.
The stain is not permanent.
Iodine green may also be used in a 0.3% aqueous solution. Amyloid is stained a violet red.

Stain for Fat.—It is obvious that unless the fat is fixed by osmic acid solution, it will be dissolved in the dehydrating agents. Therefore where fat is to be demonstrated, the tissue must be cut by the freezing method. Formalin fixation may be employed. Scarlet R., better called Sudan 4, is the stain of choice. Prepare a

saturated solution in a mixture of equal parts of 70% ethyl alcohol and acetone. The dye may be added to the solvent in a two ounce bottle until a small excess accumulates in the container on standing. After it has completely settled, pipet a small amount of the solution into the stender dish. Care must be taken not to carry any of the sediment into the staining jar for it will precipitate upon the section. When not in use, the solution must be kept in tightly stoppered bottles for the acetone evaporates quickly.

1. Stain sections for 5 minutes in the saturated solution in a stender dish.
2. Transfer with a section lifter to 70% alcohol for an instant.
3. Wash in water and counter stain in hematoxylin.
4. Wash in water.
5. Mount in glycerin jelly.

These sections are not permanent, though they be kept for months if the cover glass is rimmed with asphalt paint. Fat is stained red, nuclei blue.

Iron Containing Pigments.—Such pigments as hemosiderin may be demonstrated in fresh or formalin fixed tissue cut by the freezing method. Tissues may be embedded in celloidin. The reagent is prepared as follows:

SOLUTION A.—2% aqueous solution of potassium ferrocyanide.

SOLUTION B.—1% aqueous solution of hydrochloric acid.

These two solutions are preserved separately and mixed when ready for use. Add 1 part of solution A to 3 parts of solution B.

1. Stain sections in the mixed reagent for 20-30 minutes.
2. Wash in distilled water.
3 The section may now be stained with hematoxylin and eosin.
4. Wash in distilled water.
5 Mount in glycerin.

METHOD OF SILVER IMPREGNATION FOR GENERAL LABORATORY PURPOSES

A silver impregnating technic that is applicable to formalin or Zenker fixed tissues, that will reveal the finer fibrils as well as the other elements of a tissue, particularly tumors, is of great value to the histologist. The method given below was designed particularly to demonstrate the fibrils of tumors of the melanoma group (Foot and Foot). In making use of this stain the table should be consulted and that variant selected which will give the desired results. If one desires a selective stain of reticulum, one of the first three variants should be employed; the last three are unsatisfactory for they impregnate collagen and reticulum exactly alike, magenta to reddish. According to Foot and Foot, the second and fifth are the best for general use. The fourth or the sixth give less intense impregnations than the fifth. For delicate effects, with little disturbance of the cytoplasmic background, the first variant should be chosen; the fourth if more cytoplasmic detail, color variety and plasticity are desired. Those variants employing the tannic acid mordant will give more colorful pictures than those where it is omitted. Variant 5

Color Variations in Tissues Stained with Silver by 6 Variants

Tissue	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5	Variant 6
Nuclei	Brown	Magenta, slightly brownish	Dull magenta-brown	Brown or black	Black	Sharp brown reddish or black
Epidermal cytoplasm	Slate brown to brown	Slate violet to magenta	Rose slate, brownish to magenta	Slate brown to fuscous	Violet to violet-brown	Slate blue to slate brown
Glandular cytoplasm	Slate brown	Slate brown to magenta	Magenta-gray	Pinkish gray to brown	Pinkish gray to violet	Violet brown
Erythrocytes	Brown	Magenta	Dark brown to black	Reddish brown	Violet-brown	Brown to seal brown
Collagenous fibers	Lilac to light magenta	Deep magenta to violet	Dull magenta	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Reticular fibers	Black	Black	Dark magenta to black	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Endoneurial fibers Meissner's nevus cells	Red to black	Magenta to black	Magenta to violet or black	Red to black	Magenta to black, finest often carmine	Brick red
Skeletal muscle fibers	Slate brown striae black	Magenta to dark red, striae red to brown	Slate pink, striae red to brown	Pinkish brown striae black	Violet, striae deep magenta	Violet to black, striae indistinct, too intense
Cardiac muscle fibers	Gray	Magenta-gray	Slate pink	Gray, striae blackish	Violet, striae magenta	Violet, striae magenta
Smooth muscle fibers	Gray	Magenta	Rose-gray	Pinkish to brownish gray	Violet	Slate violet
Myelin sheaths	Black	Black	Black	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Nerve trunks	Pink to red	Magenta	Magenta	Brownish pink, epineurium darker	Magenta, epineurium darker	Brick red, epineurium grayish
Melanin	Black	Blue-black	Black	Black	Blue-black	Black

demonstrates muscle striations well. Variant 2 reveals beautifully the reticulum of the liver sinusoids, lymphatic reticulum and muscle sheaths.

The methods do not impregnate either brain or spinal cord with sufficient contrast.

Fixation.—The best fixative is neutral 10% formalin in which blocks cut thin enough to insure complete penetration of the fluid should remain 24 hours at least; longer if possible. If Bouin's fluid is used, the results are comparable to those obtained in the Laidlaw-Bouin method; the nuclei will be unimpregnated, the cytoplasm impregnated in the case of epithelial cells, and mesoblastic cells will be unstained. The resulting pictures are more colorful than those obtained by the Laidlaw procedure.

The method gives very good results if Zenker-fixed tissues are used. They should be fixed for 24 hours and washed in running water for another 24 hours. After embedding and sectioning, the mercuric chloride should be removed from the sections with the usual alcoholic iodine solution, and this in turn removed with very weak (1% or less) aqueous sodium thiosulphate. This must then be washed out thoroughly. The oxidation-reduction steps, in which the potassium permanganate and oxalic acid are used, should be omitted as they produce effects similar to Bouin's fixation. The presence of chromium salts makes no material difference in the subsequent impregnation, except to enhance the impregnation of nervous tissue. On the whole, formalin fixation gives more colorful results and is, on this account, to be preferred. This does not, however, imply that Zenker's fixation is to be eschewed—quite the contrary; it gives very striking pictures in all instances and is well suited to the method.

Embedding.—The ordinary routine method of paraffin embedding is used after dehydration of the tissue in ascending percentages of alcohol and chloroform.

Preliminary Treatment.—This is essential in the case of all the variants. The sections are de-paraffinized in two changes of xylol and absolute alcohol and are then treated from 1 to 24 hours with a mixture of 2 parts pure pyridin to one part of pure glycerol. This bath keeps well and may be used repeatedly for many weeks. The sections are transferred directly from this to 2 changes of 95% alcohol, washed in tap water and placed in distilled water.

Impregnating Fluid.—This is a simple silver diammino hydroxide solution. It is used in all the variants, at full concentration in the first three, at half strength in the last three. To 10 c.c. of 1.2% silver nitrate solution in distilled water, strong ammonia is added dropwise until the resulting brown precipitate is just dissolved; 10 c.c. of 3.2 per cent pure sodium hydroxide solution in distilled water is added and the reprecipitated silver hydroxide again just dissolved by the addition of a few more drops of ammonia. The solution is then made up to a 100 c.c. with distilled water that has been heated to about 50° C. Sections are impregnated in this in a closed staining box in the incubator at 37° C., or the paraffin oven at 55° C. for 1 hour in case of variants 1, 2, and 3, and for 10 minutes in half-strength solution (5 c.c. silver nitrate, 5 c.c. sodium hydroxide) in that of the other three variants.

Silver diamino carbonate may be used interchangeably with, and in the place of, the hydroxide; it often gives superior results, particularly in those variants in which the tannate mordant is used. It is made up at full strength in all cases; to 10 c.c. of 10.2% silver nitrate add strong ammonia drop by drop until the precipitate is dissolved. Then add 10 c.c. of 3.1% sodium carbonate in distilled water, instead of the hydroxide. There is no reprecipitation upon adding the carbonate, as the hydrogen ion concentration remains unchanged, and further ammonia is therefore unnecessary. The solution is used in exactly the same manner as the hydroxide.

Reducing Fluid.—The developer is a mixture of 1 c.c. of strong neutral formalin (40% formaldehyde); 1% sodium carbonate in distilled water 3 c.c., and distilled water to make 100 c.c. Three minutes completes the reduction.

Toning and Fixing.—The toning bath is a 1:500 solution of Merck's "acid brown" gold chloride in distilled water. The fixing fluid is the usual 5 per cent aqueous solution of sodium thiosulphate ("hypo").

VARIANT 1.—The sections are taken from distilled water, impregnated for 1 hour in the impregnating fluid, washed in 2 changes of distilled water and reduced in the developer for 3 minutes or so. They are then washed in tap water and toned for 3 or more minutes in the gold bath, washed and fixed in "hypo" solution for 3 or more minutes, after which they are washed, dehydrated in ascending percentages of alcohol, cleared in xylol and mounted in Canada balsam.

VARIANT 2.—This is similar to the preceding formula, except that the Laidlaw oxalic acid (5%) bath is intercalated between the toning and fixing baths, and the fact that toning, redevelopment and fixing are all lengthened to 10 minutes each, to correspond with Laidlaw's directions.

VARIANT 3.—In the variant, formalin-soda replaces the oxalic acid procedure of its predecessor. It is made up exactly as before (formulin 1 c.c., 1 per cent sodium carbonate 3 c.c., distilled water to 100 c.c.). Used developer should not be employed: it should be made up freshly each time. The treatment with gold, formalin and "hypo" solution is the same as in variant 2. (Instead of soda-formalin solution, a solution of 0.5% oxalic acid in 5% neutral formalin has been found to give better results and avoids the danger of precipitates.)

VARIANT 4.—In the following three variants a tannic acid mordant is used made up as follows: pure tannic acid 0.2 gram; ammonium bromide 3.5 grams; strong neutral formalin 5 c.c.; distilled water to make 500 c.c.

The sections are mordanted for 15 minutes in the tannic acid bath heated to 50° C. in the incubator or paraffin oven. They are then treated for ½ to 1 minute with 100 c.c. of distilled water to which has been added 3 to 5 drops of strong ammonia. This is the "stop" solution. They are then washed for about two minutes in distilled water. The impregnation with silver is complete at the end of 15 minutes instead of 1 hour, as in the preceding variants. After impregnation the sections are washed in distilled water. developed, toned and fixed as in variant 1.

VARIANT 5.—Proceeding as in variant 4, the method changes as soon as the toning bath is reached, to correspond with variant 2, lengthening the time to 10

minutes and using the 5% oxalic acid-gold developed in exactly the same manner.

VARIANT 6.—This resembles variant 5 in every particular except one, formalin-soda developer replaces the oxalic acid bath, as in variant 3.

The formalin-oxalic acid intensifier may be used here, as in variant 3.

Summary of Steps in the Variant.—1. Neutral formalin or Zenker's fixation.

2. Paraffin embedding.

3. Pyridin-glycerol pretreatment for 1 to 24 hours.

4. In variants 4, 5, and 6; tannic acid mordant for 15 minutes followed by "stop" solution of ammonia for 30 seconds.

5. (a) Variants 1, 2 and 3; impregnation in warm silver diamino hydroxide for 1 hour.

(b) Variants 4, 5 and 6; impregnation in this bath at half-strength for 10 minutes.

6. Reduction of silver in formalin-soda developer for 3 minutes.

7. Toning in 1:500 gold chloride in variants 1 and 4 for 3 minutes; other variants for 10 minutes.

8. Reduction of gold in variants 2 and 5 with 5% oxalic acid; variants 3 and 6 with formalin-soda; in either case for 10 minutes.

9. Fixing in 5% thiosulphate in variants 1 and 4 for 3 minutes; other variants for 10 minutes.

Note: Thorough washes are indicated between all steps, distilled water being required until the sections have been reduced in step 6; after that, tap water is employed throughout.

METHODS FOR THE PREPARATION OF SECTIONS OF BONE

Formerly bone studies were made upon extremely thin disks prepared by grinding. The same methods were applied to the study of teeth. The information gained by the study of such sections is of value chiefly to the student of normal histology rather than the pathologist. Morrell has described an especially hard knife capable of cutting fresh untreated bone in section 6-12 microns thick. With a technic so simple, as compared with the tedious and time consuming methods of grinding, perhaps more will be learned concerning the microscopic structure of bones in health and in disease. The present methods (Jaffe) deal chiefly with decalcified bone. There are a number of decalcifying agents none of which are entirely satisfactory because they cause swelling of cells or prevent subsequent differential staining.

Neutral formalin in a 10% aqueous solution is the best fixative, for swelling is less after its use than after any other fixative. Either before or after fixation the bone should be cut into slices not more than 2 or 3 millimeters thick. A thin fine hack saw blade or better still, a jeweler's saw will serve satisfactorily for small pieces but for large pieces a band saw is best. The decalcifying agent must be used in large volumes. It must be frequently agitated and changed several times during the process. It is best to decalcify in the incubator (37° C.). Decalcification must not be prolonged; its progress may be determined by sticking a fine needle into

the specimen, never by bending it. The tissue must be promptly removed and thoroughly rid of the decalcifying agent when the action is complete.

Decalcifying Agents.—Zenker's fluid because of its acetic acid content will remove small amounts of calcium during the process of fixation but it has little effect upon bone fragments. Sodium citrate in 20% solution has been recommended for many have noticed that calcareous specimens preserved in fluids containing sodium citrate become soft. Müller's fluid, like Zenker's, will decalcify if the tissue is not too dense. It is frequently used for fetal bone. It requires weeks to accomplish this purpose and fibrillar staining may be impossible after such prolonged action of bichromate.

Nitric Acid.—A 5% solution of this acid seems to be the choice of most pathologists. It is perhaps the least harmful of all decalcifying agents if used properly. A short exposure to an acid of this strength is less harmful than prolonged exposure to a weaker solution. The aqueous solution is more effective than the alcoholic. Small bubbles of gas collect about the tissue and retard the action of the acid unless the container is frequently shaken or placed in a mechanical shaker. If the bone is of the compact variety, the acid solution must be changed several times. Decalcification should be complete within 18 to 24 hours. A longer exposure to the acid will surely impair the staining qualities. After the action of the acid is complete, transfer the tissue to a 5% solution of sodium sulphate for 24 hours. During this period the sulphate solution must be frequently changed until it no longer becomes acid to litmus but remains neutral. Wash the tissue in running water for twenty-four hours after which it may be dehydrated and embedded.

Some prefer to add to the acid solution some substance that will counteract the swelling effect. Phloroglucin is recommended by Mallory. The solution is prepared as follows:

Phloroglucin .... 1 gram
Nitric acid .... 10 c.c.

The solution should be made in a 500 c.c. or liter flask for considerable heat is generated, and if a smaller vessel such as a cylinder or small flask is used the mixture will be expelled by the gaseous vapors generated. *Always keep the mouth of the flask pointed in such a direction that if the fluid is ejected it will do no harm.* After solution is complete and the fluid has cooled slightly it may be diluted with 100 c.c. of 10% nitric acid. The resulting solution is about 20% nitric acid. If a weaker solution is desired, use less acid in preparation but do not dilute the final mixture for phloroglucin is not effective in protecting the tissue in less than 1% solution.

Hydrochloric Acid.—Hydrochloric acid is used in 5% solution in 10% sodium chloride. After decalcification the tissue should be placed in 10% sodium chloride to which small amounts of lithium carbonate are added until the solution becomes neutral. After 48 hours in salt solution the tissue is washed in running water for 24 hours. This is the best method for the preservation of fibrillar structure.

Formic Acid.—In mixture with sodium citrate this acid has been recommended but in the hands of the author it has not been as satisfactory as nitric acid, and it is much more costly.

Waggoner's Solution.—This method is usually satisfactory for large or dense sections of bone. The specimen may be left in the solution for one to two months with no damage to the staining properties of the nuclei.

Fixation may be in either 10% formalin or Helly's solution but acetic-Zenker solution should not be used.

After fixation, the specimen should be washed free of the odor of formalin or over night in running water if Helly's solution has been used.

Waggoner's solution (which keeps for several months) is prepared as follows using Merck's 85% to 90% C.P. formic acid with a specific gravity of 1.2 and a molecular weight of 46.02:

Formic acid (85% to 90%) .... 50 c.c.
Distilled water .... 35 c.c.
Mix and add to following solution:
Sodium citrate (Merck C.P.) .... 17 gms.
Warm distilled water .... 85 c.c.

When the bone has been decalcified, wash in tap water to remove the odor of formic acid, embed by the paraffin or celloidin methods, remove the mercuric precipitate if fixation has been in Helly's fluid and stain as desired.

Dehydration, Embedding and Staining.—After thorough washing in running water for 24 hours, the tissue is ready for dehydration. Decalcified bone may be embedded in paraffin or celloidin. Celloidin is the better agent, because paraffin embedded bone acquires considerable hardness. The first 6 or 8 sections cut should be discarded, because the surface of the block of tissue is certain to have been lacerated by the saw or fragments of bone dust. Decalcified bone is not easily stained. One may employ a strong Delafield's hematoxylin and slightly overstain and then permit the sections to stand in water overnight. The Hansen Bock technic yields a sharp differential stain. The following solutions are required:

I. Hematoxylin .... 1 gram
   Absolute alcohol .... 10 c.c.

II. Potassium alum .... 20 grams
    Distilled water .... 200 c.c.

III. Potassium permanganate .... 1 gram
     Distilled water .... 16 c.c.

IV. Glycerin C.P. .... 50 c.c.
    Glacial acetic acid .... 50 c.c.

V. Eosin Y .... 4 grams
   95% alcohol .... 1000 c.c.

(*a*) Mix solutions I and II and then add 3 c.c. of solution III. Boil for one minute; filter when cool. This stain must be freshly prepared each time it is to be used. Stain unmounted celloidin sections in this mixture for 2-18 hours. (Paraffin

FIG. 375.—THE METHOD OF EXPRESSING MARROW FROM A RIB

The rib is thoroughly cleaned of fascia, and then, hand-over-hand, two pair of pliers squeeze the marrow from one end of the rib to the other. It emerges as shown in photograph, in thick drops, which are permitted to fall directly into the fixing solution. (Suggested by Dr. Max Lederer, Brooklyn Jewish Hospital.)

sections may be washed in xylol to remove the paraffin and then washed with alcohol and water, after which they may be handled as frozen sections and stained as above.)

(*b*) Differentiate the stained sections in solution IV. Differentiation may be

controlled by frequently floating the section on a slide and examining it with the microscope. As soon as a satisfactory result is obtained, the section should be plunged into water and washed for 1 hour. Differentiation usually requires from 5 to 20 minutes.

(c) Counter stain in solution V for 5 minutes.

(d) Dehydrate as in the routine methods, and mount in balsam.

PREPARATION AND STAINING OF BONE MARROW SECTIONS

In the preparation of bone marrow it is important to avoid dense bone that will require decalcification. When bone marrow biopsies are studied, it is well to
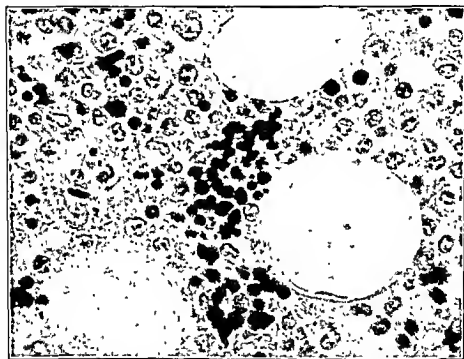
Fig 376.—Normal Sternal Bone Marrow (young adult)

About 60% cellular, the remainder fat; myelocyte erythroblast ratio from 2.1 to 6:1. A focus of erythropoiesis is seen in the center, nearly all cells being of the late erythroblast and normoblast stages. The background of large, pale cells are myelocytes, metamyelocytes and a few scattered reticulum cells.

divide the specimen into two parts. The first, the button of the bony cortex which must be decalcified, and the second the soft marrow portion which at the most contains a few spicules and does not require a special decalcifying agent. Both pieces may be fixed in Helly's fluid. The bony button must be decalcified but the soft marrow may be washed, dehydrated and embedded in paraffin. Rib marrow may be squeezed from the rib as in Figure 375. Two pair of pliers worked hand

over hand from one end of the rib to the other will force out sufficient marrow for study. The droplets may be received directly into Helly's fluid. Sections of vertebrae may be decalcified in Müller's fluid if time is not a factor. When speed is necessary, frozen sections may be made of the fixed spongy portion provided the tissue be impregnated with 12 to 15% glycerin gelatin. This requires about 5 hours at 37° C. The gelatin is allowed to cool and harden and then a block may be cut out carrying the tissue with it. Further hardening may be accomplished
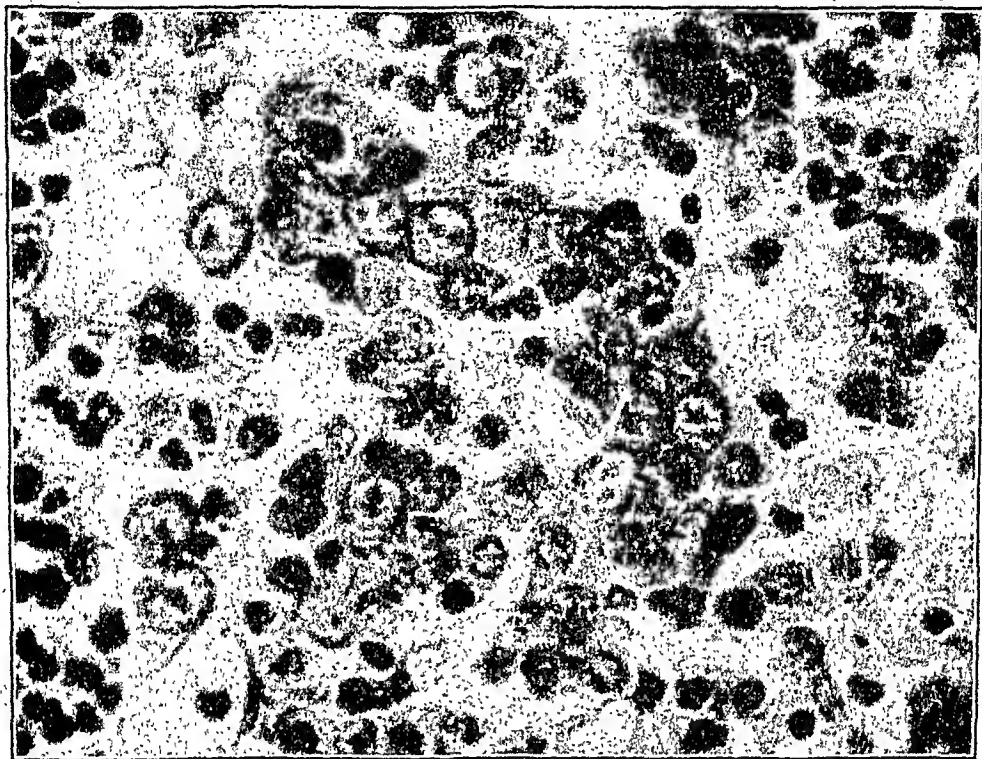
FIG. 377.—PERNICIOUS ANEMIA (RELAPSE) (sternal biopsy)

Marrow is solidly cellular and composed of a disorderly mass of megaloblasts, later forms of the red cell series being sparse and imperfect. The myeloerythroid ratio is reversed.

by soaking in 10% formalin. The block should be trimmed and well washed for at least an hour before staining.

Sections may be stained by the routine hematoxylin and eosin technic or by the methylene blue phloxine technic of Mallory. According to Schmorl the Ellerman modification of the May-Grünwald technic is the stain of choice. The method is carried out as follows:

1. Fixation in Helly's fluid containing 10% of formalin. The tissue should be obtained promptly after death.

2. Wash in running water 24 hours.

3. Dehydrate, clear and embed in paraffin, cut sections 5 microns thick.

4. After fixation on a slide and removal of paraffin, wash in water and remove

the excess water with absorbent paper. Then place in the following mixture for 15 minutes:

Eosin 1% aqueous solution .... 5 c.c.
Neutral formalin .... 0.25 c.c.

5. Wash in warm (45° C.) distilled water.

6. Stain in May-Grünwald solution (Grübler) diluted with equal amounts of water for 30 minutes.

7. Wash 5-10 minutes in distilled water. Blot dry with absorbent paper.
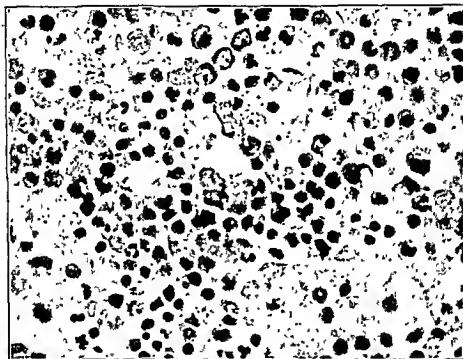
Fig. 378.—Idiopathic Hypochromic Anemia (sternal biopsy)

Marrow is totally cellular and erythropoiesis is extremely active; red cell formation is of the normoblastic type although there is a scattering of megaloblasts present. Hemoglobin content of the later nucleated red forms is exceedingly sparse, normoblasts often appearing as naked nuclei with a colorless halo.

8. Differentiate in 100% alcohol 2-4 minutes by dropping the alcohol upon the preparation until no more color comes away and the section assumes a red tone.

9. Clear in xylol and mount in balsam.

Nuclear structure of the cells of the hematopoetic system and especially the neutrophils are well stained by this method. (Figs. 376, 377, 378 and 379.)

CUSTER'S MODIFICATION OF AZURE II—EOSIN METHOD
FOR BONE MARROW [1]

This method if carefully followed yields very satisfactory sections. The various cells reveal a most striking differential stain. Experience and practice are required and the slightest variation in technic may result in failure.

1. Fix the biopsy material in formol-Zenker (Helly's) solution.
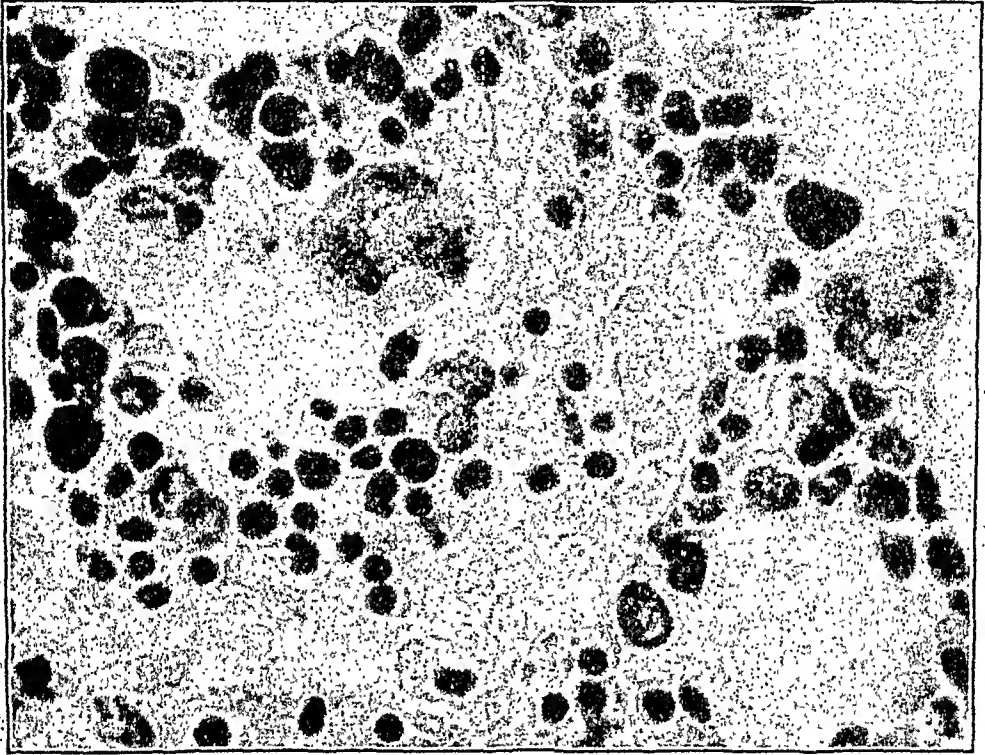
Fig. 379.—HEMORRHAGIC ANEMIA (sternal biopsy)

Cellularity is increased over the normal for the age, the majority of cells belonging to the erythropoietic series and found in the later stages of maturation. The three large cells are megakaryocytes.

2. Wash gently in running water for one hour.

3. Decalcify in Waggoner's formic-citrate mixture over night if necessary (until the bony button is soft to the prick of a needle).

4. Wash gently in running water for one hour.

5. Dehydrate in ascending strengths of alcohol.

6. Clear in chloroform.

7. Infiltrate through chloroform and paraffin and embed in paraffin.

8. Cut sections 3-4 microns thick and mount on slides in the usual manner.

9. The sections are carried through xylol, absolute and 95% alcohol as in the routine technic. The precipitated mercury is removed from the sections by im-

Cytoplasmic stain.—1. The stain is prepared as follows:

> Ponceau dexylidine (Krall; Eimer and Amend) .... 1 gm.
> Glacial acetic acid .... 1 c.c.
> Distilled water .... 100 c.c.

2. After washing in the water the sections are stained in this solution for 5 minutes, rinsed with distilled water, immersed in a 1% aqueous solution of phosphomolybdic acid solution for 5 minutes and thoroughly washed in distilled water.

3. A wider range of red colors may be obtained—varying from vermillion to ruby if two parts of the above ponceau solution are mixed with 1 part of the following:

> Acid fuchsin .... 1 gm.
> Acetic acid glacial .... 1 c.c.
> Distilled water .... 100 c.c.

The procedure is outlined above is followed.

4. If either of the above stains are diluted with 10 volumes of 1% acetic acid and the staining time lengthened to 1 hour, greater delicacy and precision of staining will result.

Connective Tissue Stain.—1. After washing in distilled water, immerse the slide in the following dish:

> Light green (Krall; Eimer and Amend) .... 2 gms.
> Glacial acetic acid .... 1 c.c.
> Distilled water .... 100 c.c.
> Add the dye to the acid water.

2. Stain for 5 minutes. The rapidity of staining varies with different samples of green.

3. Wash the preparation with 1% aqueous solution acetic acid for 2 minutes; dehydrate; clear in xylol and mount in balsam.

Nuclei will appear blue black; cytoplasm various shades of red; mucus and collagen green.

The technician is advised to consult Masson's original article for other stain combinations.

CYTOLOGIC STUDY OF VARIOUS BODY FLUIDS WITH ESPECIAL REFERENCE TO TUMOR CELLS

Frequently pleural, peritoneal, spinal and other fluids are sent to the laboratory with the request that they be examined for tumor cells. It is the custom of many to attempt to stain smears of sediment with methylene blue or some other stain. The results are so unsatisfactory that even if recognizable tumor cells are present, they would not be revealed *by this method unless they were present in large clumps.* The author conceived the idea of concentrating the cell content first by centrifuging 30 c.c. or more of the fluid if available. The supernatant fluid is poured

off with the exception of the last 1 or 2 c.c. Ten c.c. of Helly's fluid is added and the tube with its contents again centrifuged. The whole is allowed to stand for 2 to 8 hours when the sediment may be dislodged as a solid button, washed in water and carried through the usual technic of paraffin embedding. If there is abundant sediment a large round bottom tube may be used which will produce a broad disk of solidified sediment. If the sediment is scant a regular centrifuge tube should be employed.

A similar procedure may be used when splenic puncture or sternal puncture yields only a bloody fluid. Needle biopsies of tumors likewise may yield fragments too tiny to be handled individually. Where salt solution washings carry the cells to be studied or whenever the fluid is known to be poor in proteins, it is well to pour off all the supernatant fluids after the first centrifuging and to add 1 to 2 c.e. of clear serum before adding the Helly's fluid.

By this method, not only are the cells concentrated, but they are seen as one customarily sees tumor cells. Frequently the cells obtained in pleural or peritoneal fluids of malignant disease may be found to compare amazingly well with the cells of the tumor seen in paraffin sections.

PREPARATION OF MUSEUM SPECIMENS

No matter how small an institution or its laboratory may be, there should be an attempt made to preserve good pathological specimens. Good museum technic is acquired only with practice (Figure 380). The main object is to mount a specimen in such a manner that all of its features may be apparent. Specimens in jars should show the original color and surface texture. Consistency cannot always be preserved. The various fixing agents harden the tissue. Soft tissues, that are frequently handled, soon fall to pieces; so while it is well to keep the more common lesions as "wet" specimens in large crocks, the uncommon ones should be mounted in suitable museum jars.
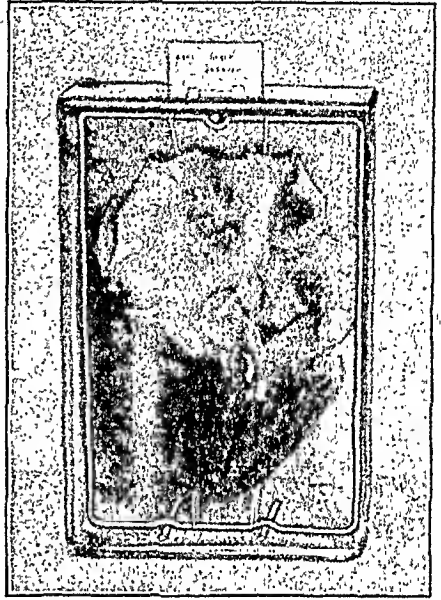
FIG. 380.—A HEART PROPERLY MOUNTED AND READY FOR THE MUSEUM

Note the position of the glass frame and its feet one at the top and two at the bottom which serve to keep the frame in the center of the jar. Note the position of the linen threads which support the specimen. They are efficient yet not unsightly. Note also the position of the label. (From Gradwohl, *Clinical Laboratory Methods and Diagnosis*, C. V. Mosby and Co., St. Louis, Mo.)

Selection of Material for the Pathological Museum.—Representative material is obviously the most desirable for the medical museum is primarily a teaching unit and not a collection of curiosities. Material should be placed in a fixing solution as soon after removal from body as possible. Autopsy material must be

fixed very shortly after death. Postmortem changes alter the appearance and consistency of organs; *exposure to air is certain to affect the color.*

Fixation. Thorough fixation by a properly prepared fluid is necessary. Large organs like the lung and liver or brain do not fix well. The same is true of huge spleens from cases of leukemia or large tumors. It is far better to preserve slices of these organs which will show the pathology well. Such specimens cost less to prepare and are more easily handled. The basic principles of preparation are herein laid down. More detailed accounts may be obtained by consulting the Journal of Technical Methods issued by the International Association of Medical Museums.

Kaiserling Method.—This is by far the oldest and most popular method in use today. It is also the most tedious. The organ should be suspended or laid in absorbent cotton in the position in which it will best reveal the lesion. Pieces of cotton should be inserted between opposing surfaces that touch so that the fluid may easily reach all parts. It must then be covered with an amount of fluid equal to about 10 times its volume. Intestine requires about 24 hours for complete fixation. Thin organs, such as kidney, if cut through require 4 to 5 days. Spleen, liver, or lung may require weeks. It is better to cut slabs of the latter organs, not more than 4 cms. thick. Kaiserling fixing solution is prepared as follows:

Potassium acetate	170 grams
Potassium nitrate	90 grams
Formalin (neutral)	1600 c.c.
Water	8000 c.c.

Only the best chemicals should be used. Formalin may be neutralized with sodium hydroxide or marble block.

Color Restoration.—After fixation as outlined above the specimen will appear to have lost much of its color. It must be thoroughly washed in running water for about 24 hours or until it has lost the odor of the formalin. It is now placed in 95% alcohol. The color will gradually return. This step must be carefully watched, for after reaching its full intensity the color will fade again. Alcohol solutions lose their strength with use. The concentration of alcohol should never be permitted to fall below 80%.

Preservation.—Wash the specimen again in running water and then place it in the preserving fluid prepared as follows:

Potassium acetate	1815 gms.
Glycerin	2000 c.c.
Distilled water	10,000 c.c.
Carbolic acid (preservative)	20 c.c.

If the preserving fluid becomes cloudy it is best filtered with fine animal charcoal. Add one heaping tablespoonful of charcoal to each 2000 c.c. and filter through filter paper in a large (2000 c.c.) funnel. It may be necessary to pour the first filtrate back into the funnel.

Klotz Method.—This is less complicated and gives excellent results provided sufficient fluid is used. Solution I must be used in volumes 10 times as great as the organs being fixed and the solution must be changed when it becomes cloudy. Solution I for fixation is prepared as follows:

Carlsbad salts (artificial)	1750 gms.
Chloral hydrate	1750 gms.
Formalin	1750 c.c.
Water	35,000 c.c.

About the same amount of time is required for fixations as with the Kaiserling method. After fixation, wash the specimen in running water for 24 hours. It is then placed in Klotz solution No. 2 prepared as follows:

Carlsbad salts (artificial)	875 gms.
Chloral hydrate	350 gms.
Formalin	175 c.c.
Water	35,000 c.c.

This is the final preserving solution. After several days or weeks, the specimen should be placed in fresh clear preservative and sealed in a suitable jar.

The formula of Klotz and MacLachlan for making artificial Carlsbad salts is as follows—

Sodium Sulphate	22· grams
Sodium Bicarbonate	20 grams
Sodium Chloride	18 grams
Potassium Nitrate	38 grams
Potassium Sulphate	2 grams

Selection of Jars.—The square type jar is obtainable in many sizes. The cheaper domestic jars frequently show irregularities in the refraction of light giving a wavy effect. These jars should be rejected. Carefully selected domestic jars planed and polished on one surface are as satisfactory as the more expensive imported jars. Four sizes should be available. One that will conveniently hold a large heart (14 × 12 × 9 cms.); a kidney jar (17 × 12 × 5 cms.); intestine jar (32 × 9 × 5 cms.) and a small specimen jar (16 × 10 × 5 cms.).

Glass frame should be made to fit each jar snugly. For the jars listed above glass rods about 6 to 7 mm. diameter make the best frames. Soft glass is the more easily handled and just as serviceable as hard glass. The inside measurement of the jar should be carefully laid out as a rectangle on a sheet of asbestos. The rod should be marked with a wax pencil at the points where it is to be bent. Heat the glass with a blow torch at the first mark until it is red hot and soft. It may then be placed upon the asbestos and bent along the lines drawn. Each angle is bent until the rectangle is completed. Feet or cross bars must be fused on the frame so that it will remain in the desired position in the jar. These must be so placed that they support no weight but only prevent lateral

motion of the frame. The entire frame must fit snugly within the jar. After the frame is completed it is well to heat a corner to red heat so that the frame may accommodate itself for the contraction which accompanies cooling.

Specimen should be fastened to frames with white linen thread. The thread must pass through fibrous connective tissue, as that which surrounds blood vessels, lest it pull or cut through. Small celluloid buttons may be used to prevent cutting by thread. One must always suspend the specimen with care and thought so that the lesion shows to the best advantage while the organ is seen in a natural anatomic position.

Labels must be attached to every specimen before mounting. The most satisfactory label is made by marking identification numbers upon linen tape with waterproof ink. The tape is then soaked in hot melted paraffin for 10 to 15 minutes. Such a label will last indefinitely.

After the specimen has been placed in the jar, it may be covered with the preserving fluid and then allowed to stand for several days, covered but not sealed. Air bubbles are certain to collect on the specimen or upon the sides of the jar. These may be dislodged with a small brush. The jar may now be sealed with asphalt cement. Trinidad lake asphalt has seemed the most satisfactory according to Muir and Judah. The cement must be heated on a sand bath until it has a liquid consistency. The cover of the jar should be pierced by a small hole to permit final filling of the jar with fluid. The cover is heated in an oven until quite hot. The glass surfaces must be free of grease. Heat the top of the jar gently and then cover the edges with cement. Heat the cement on the jar edges and then place the cover in position and press it down firmly. While the cement is hot scrape off the excess. Allow the cement to harden and then fill the jar through the small hole in the cover. This hole may be plugged with a piece of cork or with sealing wax.

The specimen may now be completed with a suitable descriptive label which will indicate clinical and laboratory reference numbers, the source of the material, a short history of the case, microscopic findings and final diagnosis.

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APPENDIX

DONATH-LANDSTEINER TEST FOR PAROXYSMAL HEMOGLOBINURIA (MacKenzie's Modification)

Principle.—In paroxysmal hemoglobinuria there is present in the blood a thermolabile autohemolysin which is responsible for the destruction of sufficient erythrocytes to cause the symptoms characteristic of this disease.

Procedure.—1. Obtain blood by venous puncture. Add a small portion to 10 volumes of 0.85% saline solution. The remainder is placed in a dry tube and allowed to clot. The syringe, tubes and saline solution should be warm and the blood kept warm during the preparation of the serum and cell suspension.

2. Wash the cells three times in warm 0.85% saline solution and prepare a 5% suspension.

3. Centrifuge the clotted blood and remove the serum as soon as possible after clotting.

4. Obtain blood in the same manner from a normal individual of the same blood group as the patient (control) and prepare serum and a 5% suspension of erythrocytes in the same manner.

5. Prepare a 1:10 dilution of fresh guinea pig serum (complement).

6. Set up the test as follows:

Tube	Patient's serum	Patient's R.B.C. 5%	Control Serum	Control R.B.C. 5%	Comp. 1:10	Saline 0.85%	
1	0.25 c.c.	0.1 c.c.	....	....	0.1 c.c.	0.05 c.c.	Immerse in
2	....	....	0.25 c.c.	0.1 c.c.	0.1 c.c.	0.05 c.c.	ice water
3	0.25 c.c.	....	....	0.1 c.c.	0.1 c.c.	0.05 c.c.	—10 min.
4	....	0.1 c.c.	0.25 c.c.	....	0.1 c.c.	0 05 c.c.	Water-bath
5	....	0.1 c.c.	....	....	0.1 c.c.	0 3 c.c.	at 37° C.
6	....	....	....	0.1 c.c.	0.1 c.c.	0.3 c.c.	—30 min.

7. Set up duplicate test in exactly the same manner except that chilling in ice water is omitted.

8. A positive reaction is indicated by hemolysis in tubes 1 and 3 of the first set chilled in ice water for 10 minutes followed by water bath at 37° C. for 30 minutes. There should be no hemolysis in the second or unchilled set.

METHOD FOR QUANTITATIVE DETERMINATION OF PROTHROMBIN (Quick)

Principle.—The coagulation time of blood or plasma can be employed as a measure of the prothrombin concentration, if the other factors in the clotting

process are made constant. The coagulation mechanism is concisely presented in the form of two equations:

$$\text{Prothrombin} + \text{thromboplastin} + \text{calcium} = \text{thrombin.}$$
$$\text{Fibrinogen} + \text{thrombin} = \text{fibrin.}$$
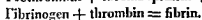
Since the clotting time is proportional to the concentration of thrombin, one can assume that it is also proportional to the concentration of prothrombin provided thromboplastin, calcium and fibrinogen are made constant, for under these conditions the amount of thrombin formed is dependent upon the concentration of prothrombin present in the blood. The method consists essentially in adding to oxalated plasma, an excess of thromboplastin and then recalcifying with a fixed quantity of calcium chloride. The coagulation time is a direct measure of the concentration of prothrombin and can be evaluated by referring to a chart which was made by plotting the clotting time for known concentrations of prothrombin.
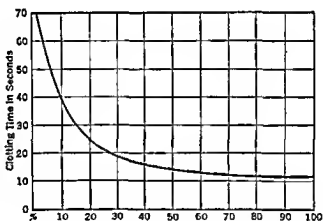
CONCENTRATION OF PROTHROMBIN IN PLASMA (HUMAN)

The relationship of the clotting time of recalcified plasma (with excess thromboplastin) to the concentration of prothrombin.

Reagents.—1. 0.10 M of sodium oxalate. Dissolve 1.34 gram of C.P. anhydrous sodium oxalate in 100 c.c. of distilled water.

2. 0.025 M of calcium chloride. Dissolve 1.11 gram of C.P. anhydrous calcium chloride in 400 c.c. of distilled water.

3. Thromboplastin. About 0.3 gram of dehydrated rabbit brain is thoroughly mixed with 5 c.c. of a freshly prepared physiological sodium chloride solution and incubated at 50° C. for 10 to 15 minutes. The milky supernatant liquid is used which is obtained after the coarse particles have been removed either by very slow centrifugation or by spontaneous sedimentation.

The rabbit brain is prepared by completely removing all blood vessels, i.e., stripping of the pia, and then macerating the brain in a mortar under acetone. By replacing the acetone several times, a non-adhesive granular powder is obtained which is dried on a suction filter. The material is then placed in small ampules which are evacuated for 3 minutes by means of an oil vacuum pump and then sealed. Such a preparation retains its full activity apparently indefinitely.

Procedure.—1. By venipuncture 4.5 c.c. of blood are drawn and mixed immediately with 0.5 c.c. of 0.1 M sodium oxalate. Plasma is obtained by centrifugation.

2. Into a test tube immersed in a water bath at 38° C. place 0.1 c.c. of plasma and then add 0.1 c.c. of thromboplastin. Allow about 30 seconds for contents of tube to come to water bath temperature, then quickly add 0.1 c.c. of 0.025 M calcium chloride. The time from the addition of the calcium chloride to .the formation of the clot is accurately recorded with a stop watch. With an active preparation of thromboplastin, the coagulation time should be 11½ to 12½ seconds for normal plasma. By means of the accompanying chart, the prothrombin concentration can be readily evaluated.

METHODS FOR THE EXAMINATION OF SEMEN

Principle.—The purpose of the examination of semen is to determine whether, in cases of sterility. the cause is due to quantitative or qualitative changes in the spermatozoa.

Collection of Semen.—1. A rubber condom is thoroughly washed under running water and dried before use.

2. After ejaculation the semen is transferred immediately from the condom to a clean glass container such as sputum bottle or test tube. This facilitates transportation and prevents any deleterious effect of the rubber on spermatozoa, which is particularly the case when the condom contains spermicidal substances. The container should be tightly closed with a cork stopper and labeled with name, date and hour of emission.

3. The specimen should be delivered to the examiner as quickly as possible. Precautions to keep the sample at 37° C. are unnecessary and may even prove harmful if patients, told to keep the specimen warm in a thermos bottle. do not measure the exact temperature. Furthermore, motility lasts longer at lower temperature, allowing more time for transportation.

Macroscopic Examination.—1. Measure the volume of semen in a small graduated cylinder. The amount varies between a few drops up to 10 c.c. There is quite a variation in amount in different samples from one donor. depending on the period of continence preceding the examination. The average lies between 3 and 4 c.c. Samples amounting to less than 1.5 c.c. are considered below normal, though sterility could not be ascribed to this fact alone unless other deficiencies are recognized in the same specimen.

2. Note the viscosity of the sample. Freshly ejaculated semen acquires a high degree of viscosity but self-liquifaction takes place and should be completed after 30 minutes. *The absence of liquifaction may inhibit the movement of spermatozoa thereby interfering with fertilization.*

3. The determination of the pH is of little value. It is always found to be on the alkaline side with a range from 7.2 to 8.9, the average being 7.8. Abnormalities never are accompanied by a fall below 7.2.

Motility of Spermatozoa.—1. Place a drop of semen on a slide and cover with a cover glass or prepare a hanging drop. Examine with low and high dry objectives. The following should be noted:

Azoospermia: absence of spermatozoa (this should be confirmed after centrifugation of specimen).

Oligozoospermia: only a few motile spermatozoa found.

Necrozoospermia: spermatozoa are present but immobile.

Observe also the presence of cells other than spermatozoa, i.e., testicular cells, epithelial cells, leukocytes or erythrocytes. The presence of crystals should also be noted.

2. If motile spermatozoa are found, note roughly the proportion of motile to immobile cells. For this determination the following technic is recommended: a disc of back paper is fitted in the eyepiece of the microscope after cutting a small slit or square in the middle. This limits the field of vision and thus simplifies the counting of the spermatozoa and decreases errors introduced by the constant change of the sperm population in the field. Immobile spermatozoa are counted after the motile ones.

3. Ten to fifteen per cent of immobile spermatozoa are encountered in fertile samples. *If the motility is very low,* repeat the examination after heating the sample to 37° C. This frequently restores motility.

4. A differentiation between "locomotoric" and "stationary" or other grades of motility is without great value since spermatozoa are known to stick to interfaces such as the surface of the drop or the cover glass, or they may attach themselves to formed elements such as cells. Furthermore, the viscosity of the sample may influence the motility. There is considerable individual variation in the ability of spermatozoa from different specimens to remain motile, which partly depends on the temperature at which the sample is kept. At 37° C. all spermatozoa usually are immobilized after 8 hours while at 4° C. a number of motile cells may be encountered after as many as 4 days or more. To determine the viability of a specimen it is, therefore, advantageous to keep it at 4° C. and to determine the motility in samples taken after 6, 12 and 24 hours.

Counting of Spermatozoa.—The number of spermatozoa in a sample of semen are counted in the same manner as described for leukocytes except for the diluent.

1. Mix the specimen thoroughly by very gently shaking or stirring with a glass rod.

2. Using a leukocyte counting pipet, draw the semen up to the 0.5 mark and the diluent to the mark 11:

DILUTING FLUID

Sodium bicarbonate .... 5 gm.
Formalin .... 1 c.c.
Distilled water ....100 c.c.

Note: The bicarbonate in the diluent counteracts mucus and the formalin immobilizes the spermatoza. When the specimen is very viscous due to an excess of mucus, 1 c.c. of semen in 19 c.c. of diluting fluid thus omitting the use of the leukocyte pipet. In case the number of spermatozoa in a sample proves to be very small, it will be necessary to draw the seminal fluid to the mark 1.0 in the leukocyte pipet, or 1 c.c. is added to 9 c.c. of the diluent.

3. Fill the chamber of the hemocytometer with the diluted specimen, count and calculate as described on page 76 for counting leukocytes. The result is expressed in the number of spermotozoa per cubic centimeter. It is therefore necessary to multiply the result by 1,000, since leukocytes are calculated in cells per cubic millimeter.

4. The normal semen contains an average of 100 to 150 million spermatozoa per cubic centimeter. The lower the count below 60 million the less likelihood of fertility. However, no diagnosis of sterility is justified unless other abnormalities are found in the sample.

Method for Staining Seminal Smears.—1. Take a clean slide and prepare a thin smear of semen.

2. Dry in air and fix by heat.

3. Add 1 per cent chloramine for several minutes to remove excess mucus.

4. Wash with water followed by 95 per cent alcohol. Dry by blotting on filter paper.

5. Stain for 2 to 5 minutes with the following:

Ziehl Neelsen's carbol fuchsin .... 2 parts
Conc. alcoholic sol. of eosin .... 1 part
Alcohol 95% .... 1 part

6. Wash with water and counterstain with Loeffler's methylene blue for a few seconds.

7. Wash, dry and examine under oil immersion.

8. The heads of the spermatozoa show a purplish color while the tail and middle piece are red.

Morphological Examination for Immature and Abnormal Spermatozoa.—The purpose of this examination is to discover abnormal forms of spermatozoa and to estimate their proportion to normal cells. Cellular constituents other than spermatozoa should be noted. Stained smears are employed.

1. Count the number of spermatozoa in a microscopic field without regard to their morphology.

2. The same field is searched for immature forms of spermatozoa. The different

developmental stages may be encountered in pathological semen but are rare in fertile specimens.

3. Examine 100 to 500 spermatozoa for abnormalities referable to the *heads* (which may be too small or too large, pointed or with ragged edges, show an atypical distribution of chromatin, the presence of acidophil vacuoles or double heads); the *midde* pieces (which may be absent, bifurcated, swollen, etc.) and the *tails* (which may be double, curled, rudimentary or absent).

4. Semen containing up to 20 per cent abnormal spermatozoa is still considered fertile. The higher the percentage of abnormal cells above this arbitrary value, the more doubtful the fertility.

5. Finally, examine for cells other than spermatozoa, i.e., epithelial cells, leukocytes and erythrocytes. Also crystals may be found in abundance. However, the age of the sample has to be considered since numerous crystals may be formed in normal semen upon standing.

Biometrical Evaluation of Spermatozoa.—This study has the objective of measuring the length of the head of a large number of spermatozoa for the establishment of a distribution curve. While in fertile cases this curve shows the high peak and small base of a "normal" distribution curve it takes on an irregular and flatter shape with broader base when infertile cases are examined. It is this irregularity or flatness of the distribution curve which is significant, and not the average head length in the individual samples. This may vary in fertile samples between 3.8 and 5.4 microns. The method is as follows:

1. A stained seminal smear is prepared according to the method described.

2. The side is focused under oil immersion. The magnification should reach at least 1500X. The microscope should be fitted with a mechanical stage.

3. An inclined mirror is fixed to the eyepiece of the microscope to project the image onto a screen consisting of a revolving plate of 10-inch diameter. The screen is covered with white graph paper in millimeter scale (added enlargement 3000X total).

4. The individual spermatozoön is measured by turning the screen in such a way that one of the lines of the graph paper parallels the axis of the cell. The length of the head can be read off directly from the paper.

5. Three hundred cells are measured in such a way and after grouping of the heads showing approximately equal length, the distribution curve is plotted, or the coefficient of variation is calculated. The coefficient of variation will be greater in infertile samples than in fertile samples.

Detection of Semen on Materials for Medicolegal Purposes.—In medicolegal cases seminal stains on materials such as clothing, linen, etc., may be identified by demonstrating the presence of spermatozoa, or by microchemical reactions such as the Florence test. Hektoen and Rukstinat have reported upon the use of a precipitation test using material from such stains as antigen. They showed also that the blood group of the donor might be determined.

Demonstration of Spermatozoa.—1. A small piece of the soiled material, not more than one-half inch in diameter, is placed on a clean slide. It is wetted

with a few drops of saline solution and the surface scraped off with a scalpel. The few drops of fluid are then spread over the slide and after drying the film is stained according to the method described.

2. Examine the slide for the presence of spermatozoa and note any abnormal forms; these may be helpful in identifying a sample since the semen from a given individual may present characteristic abnormalities.

Microchemical Method (Florence Test).—In cases where an azoospermia is present, no spermatozoa can be obtained from suspected stains. but a positive microchemical test will be a strong suggestion that the stain in question is of seminal origin.

1. Soften the material with water and place upon a slide.
2. Add a few drops of reagent:

<div align="center">

REAGENT

Iodine .... 2.54 gm.
Potassium iodide .... 1.65 gm.
Distilled water ....30.0 c.c.

</div>

3. Examine at once with the medium power of the microscope.

4. If the stain is produced by semen there will be found dark brown crystals in the form of rhombic platelets which resemble hemin crystals, or of needles, often in clusters.

5. These findings are not absolute proof of the presence of semen as some other substances give the same reaction among which are crushed insects and extracts of various organs. The reaction will occur even though the semen is several years old.

METHOD FOR THE DETERMINATION OF UROBILINOGEN IN THE FECES AND URINE FOR LIVER FUNCTION

The method of Sparkman for the determination of urobilinogen in the feces and urine is one of the most promising of the liver function tests now available.

Principle.—The determination depends on the so-called aldehyde reaction, with development of a red color on addition of an acid solution of paradimethylamino-benzaldehyde (Ehrlich's aldehyde reagent) to solutions containing urobilinogen. Comparison is made with artificial standards prepared from gold chloride and sodium bromide.

Reagents.—1. The *aldehyde reagent* is prepared by adding to 75 c.c. of hydrochloric acid an equal amount of distilled water and 10 gm. paradimethylamino-benzaldehyde.

2. 4 per cent solution of *gold chloride.*
3. 10 per cent solution of *sodium bromide.*

Standards.—1. Strong standard = 1 vol. sodium bromide, 1 vol. gold chloride, made up to 15 vol. with distilled water. Computed final value 8.2 mg. urobilinogen per 100 c.c.

2. Intermediate standard = 1 vol. strong standard diluted with 1 vol. of distilled water. Final value equivalent to *2.4 mg. urobilinogen per 100 c.c.*

3. Weak standard = 1 vol. intermediate standard diluted with 1 vol. of distilled water. Value equivalent to *0.9 mg. urobilinogen per 100 c.c.*

Procedure for Fecal Urobilinogen.—1. Transfer 5 gm. of the mixed stool to a mortar, emulsify in 100 c.c. distilled water, and transfer to a 250 c.c. Erlenmeyer flask.

2. Dissolve 8 gm. crystalline ferrous sulfate in 40 c.c. of water and mix with emulsion.

3. Add slowly 40 c.c. of 10 per cent sodium hydroxide, with rotation of flask. Stopper and shake.

4. Place in water bath for fifteen minutes at 50° C. Cool to room temperature, then filter (Whatman No. 2).

5. Pipet 5 c.c. of filtrate into test tube. Add 5 c.c. distilled water, 0.3 c.c. of five times normal HCl, and 1 c.c. Ehrlich's reagent. Allow five minutes for full development of color.

6. Compare in colorimeter with nearest standard and calculate as follows:

(1) *Strong standard:*

$$\frac{\text{Reading Standard}}{\text{Reading Unknown}} \times 630 = \text{mg. of urobilinogen per 100 gm. of stool.}$$

(2) *Intermediate standard:*

$$\frac{\text{RS}}{\text{RU}} \times 185 = \text{mg. urobilinogen per 100 gm. of stool.}$$

(3) *Weak standard:*

$$\frac{\text{RS}}{\text{RU}} \times 70 = \text{mg. urobilinogen per 100 gm. of stool.}$$

Procedure for Urinary Urobilinogen.—Single samples should be freshly voided. Twenty-four-hour specimens are collected in brown glass bottles containing 100 c.c. purified petroleum benzine and 5 gm. anhydrous sodium carbonate.

1. To a small portion of urine in a flask, add anhydrous calcium chloride in the proportion of 50 c.c. of urine to 2 gm. of the salt. Mix well and filter.

2. Test residue for bile pigment. Pour a few drops of concentrated nitric acid down side of filter. A colored zone consisting of a central pink area with a green periphery indicates the presence of bilirubin.

3. To 10 c.c. of filtrate add 1 c.c. of Ehrlich's reagent and invert several times. Allow five minutes for full development of color.

4. Match sample promptly in colorimeter. If concentration is unusually high, repeat procedure after suitable *dilution* of the specimen and calculate as follows:

(1) *Strong standard:*

$$\frac{\text{R Standard}}{\text{R Unknown}} \times 8.2 = \text{mg. per 100 c.c. of urine.}$$

(2) *Intermediate standard:*

$$\frac{RS}{RU} \times 2.4 = \text{mg. per 100 c.c. of urine.}$$

(3) *Weak standard:*

$$\frac{RS}{RU} \times 0.9 = \text{mg. per 100 c.c. of urine.}$$

Interpretation.—*Normal values* given by Sparkman are between 150 and 300 mg. of urobilinogen per 100 gm. of stool, with extreme limits of 70 to 600 mg. Any specimen of urine yielding a color in the range of the weak or intermediate standard may be regarded as not containing increased amounts of urobilinogen, and should be reported as "not increased." Colors in the range of the strong standard should be regarded with suspicion. Values of over 8 mg. per 100 c.c. of urine almost certainly represent pathological urobilinogenuria:

	Urinary Urobilinogen	*Fecal Urobilinogen*
Obstructive jaundice	Not increased	Decreased
Hepatogenous jaundice (without liver cell disease)..	Not increased	Great increase
Portal cirrhosis, hepatitis, anemia (liver cells affected)	Decidedly increased	Decreased
Catarrhal jaundice	Early increased, later decreased (intrahepatic obstruction), then increased	Decreased at height of disease

METHOD FOR THE DETERMINATION OF HIPPURIC ACID IN URINE FOR LIVER FUNCTION

Principle.—Quick has observed that the hourly rate of excretion of hippuric acid in normal persons following the ingestion of sodium benzoate is remarkably constant while in certain types of disease of the liver it is markedly reduced. This reduction is due primarily to the diminished capacity of the liver to synthesize amino-acetic acid and in part to damage of the enzymatic mechanism which unites benzoic acid with amino-acetic acid. The output of hippuric acid after the ingestion of benzoic acid is considered a measure of the liver's capacity to furnish amino-acetic acid and an index of its detoxifying power.

Procedure.—1. One hour after a light breakfast of coffee and toast the patient is given 6 gm. of sodium benzoate dissolved in 30 c.c. of water, preferably flavored with oil of peppermint. This is followed by one-half glass of water. Immediately after taking the drug the patient voids, and he then collects complete specimens of urine hourly for four hours. Should the analysis be delayed more than ten hours, the samples are preserved with toluene.

2. The specimen for each hour is measured and transferred to a beaker. If the volume of any specimen exceeds 100 c.c., it is acidified with a few drops of acetic acid and concentrated in a water bath to about 50 c.c. Each of the four specimens is acidified with 1 c.c. of concentrated hydrochloric acid and then tested with congo red paper. If the paper does not turn blue, more acid must be added. Each specimen is vigorously stirred until the precipitation of hippuric acid is complete and then is allowed to stand for one hour at room temperature.

3. The crystalline hippuric acid is filtered off on a small filter plate or Buchner funnel, washed with a small quantity of cold water and allowed to dry in the air. The dry samples of hippuric acid are weighed (to the second decimal place is sufficiently accurate). If a balance is not available, the precipitate may be dissolved in hot water and titrated with two-tenths normal sodium hydroxide, phenolphthalein being used as an indicator (1 c.c. = 0.0358 gm. of hippuric acid). To the amount of hippuric acid determined by weight or titration one adds the amount remaining dissolved in the urine. This can readily be calculated since 100 c.c. of urine at room temperature will dissolve 0.33 gm. of hippuric acid. Thus, for example, if the hourly specimen amounts to 70 c.c. and the amount of hippuric acid obtained by weight is 1.1 gm., the total will be 1.1 plus $(0.33 \text{ gm.} \times \frac{70}{100})$ or 1.33 gm. of hippuric acid. To express this result in terms of benzoic acid, one multiplies by the factor 0.63. Therefore, 1.33 gm. $\times$ 0.63 equals 0.91 gm. of benzoic acid.

Interpretation.—The normal adult will excrete about 3 gm. of benzoic acid in the form of hippuric acid in four hours. Therefore, 3 gm. has been taken as the normal value for calculating the efficiency of the liver. On this basis the normal range is from 85 to 110 per cent. This provides for the variation due to the size of the individual patient.

METHOD FOR THE DETERMINATION OF SERUM LIPASE FOR PANCREATIC DISEASE

As shown experimentally by Cherry and Crandall, lipase appears in the blood after injury to the pancreas and the determination of serum lipase has been employed for the diagnosis of acute pancreatitis and carcinoma of the organ associated with painless jaundice. The method given is that of Cherry and Crandall slightly modified by Comfort and Osterberg. Others have found the determination of serum amylase or diastase satisfactory for the same purpose and especially since it can be completed in about 2 hours.

Principle.—The substrate used in the test is an emulsion of olive oil. The degree of lipase activity is indicated by the amount of fatty acid liberated, and is reported in terms of cubic centimeters of N/20 sodium hydroxide solution.

Reagents.—1. Emulsion of olive oil. This should be purchased from a commercial laboratory. It is prepared in a homogenizer from equal parts of pure olive oil, free from fatty acid, and a 5 per cent solution of acacia, with 0.2 per cent of sodium benzoate added as a preservative.

2. Phosphate buffer solution. pH 7, Prepare Sörenson phosphate buffer solution from a fifteenth-molar solution of primary potassium phosphate. and fifteenth-molar, secondary sodium phosphate solution. adjusted to pH 7.

3. Twentieth-normal solution of sodium hydroxide.

4. A 1 per cent solution of phenolphthalein in alcohol.

5. Ethyl alcohol (95 per cent).

Procedure.—1. Place 1 c.c. of serum in a test tube. Add 2 c.c. of olive oil emulsion. 3 c.c. of distilled water and 0.5 c.c. of buffer solution. Shake the mixture and incubate at 38° C. for twenty-four hours.

2. Prepare a "blank." Place 1 c.c. of serum in a test tube. Add 3 c.c. of distilled water. Heat to 70° C. for about five minutes to destroy the enzyme. Add the buffer and substrate. Shake the mixture and incubate at 38° C. for twenty-four hours.

3. After twenty-four hours, add 3 c.c. of alcohol to each tube. Titrate to a permanent pink color of the indicator with twentieth-normal solution of sodium hydroxide.

Subtract the number of cubic centimeters of sodium hydroxide used to neutralize the "blank" (tube 2), from the number of cubic centimeters used to neutralize the acidity in tube 1, and record the difference as cubic centimeters of twentieth-normal solution of sodium hydroxide for each cubic centimeter of serum.

Interpretation.—According to Comfort the normal range is from 0.2 to 1.5 c.c. of N/20 solution of sodium hydroxide for each cubic centimeter of serum. A sharp rise. even up to 9 to 10 c.c.. may occur in acute pancreatitis and other injuries to the gland.

SIMPLIFIED KOLMER COMPLEMENT FIXATION TEST

This test is conducted in exactly the same manner as described for the quantitative test except that one dose of *serum* (0.2 c.c. with 0.2 c.c. in the serum control) is employed. With *spinal fluid* a single dose of 0.5 c.c. with 0.5 c.c. in the control is employed. In other words, the technic is exactly the same insofar as the preparation and titration of hemolysin, complement. and antigen are concerned as likewise the inclusion of antigen, hemolytic system. and corpuscle controls. When large numbers of tests are conducted, controls with known positive and negative serums are not required: but otherwise, and especially in the case of inexperienced serologists, they should be included. The test is not as sensitive as the quantitative test (page 628) or the qualitative test (page 630).

1. Preliminary removal of natural antisheep hemolysin is omitted.

2. Heat the serums in a water bath at 55° to 56° C. for 30 minutes. In the quantitative test, heating is for 15 to 20 minutes only and is preferred in order to reduce to a minimum the destruction of antibody, but 30 minutes may be employed so that the serums are prepared in the single operation for the Eagle, Hinton, Kahn, or Kline flocculation tests. Spinal fluids do not require heating unless kept for more than 3 days at room temperature (as during shipment in the mails) when

heating at 55° to 56° C. is advisable for the removal of thermolabile anticomplementary substances.

3. For each serum arrange two test tubes and place 0.5 c.c. of saline solution in the second or serum control tube.

4. For each spinal fluid arrange two test tubes; no saline solution.

5. Place 0.2 c.c. of serum or 0.5 c.c. of spinal fluid in each of the two test tubes.

6. Add 0.5 c.c. of antigen, carrying the optimum dilution, in the first tube of each set.

7. Wait 10 minutes at room temperature when 1 c.c. of complement (carrying two full units) is added to all tubes.

8. Primary incubation in a refrigerator at 6° to 8° C. for 15 to 18 hours followed by 10 to 15 minutes in a water bath at 37° C.

9. Add 0.5 c.c. of hemolysin (carrying two units) and 0.5 c.c. of 2 per cent suspension of washed sheep corpuscles to all tubes.

10. Secondary incubation in a water bath at 37° C. for 1 hour when readings are made, although experienced serologists are advised to remove the racks and make readings 10 minutes after complete hemolysis of the serum antigen, and hemolytic system controls.

The reactions may be reported as positive, doubtful, or negative, as recommended by the Committee on the Evaluation of Serodiagnostic Tests for Syphilis of the United States Public Health Service cooperating with the American Society of Clinical Pathologists, as follows: *Positive* $++++$ (4), $+++$ (3), $++$ (2) or $+$ (1), in the first tube. *Doubtful:* $\pm$ in the first tube. *Negative:* — in the first tube. However, Kolmer recommends reporting as strongly positive, weakly positive, doubtful, and negative as follows: *Strongly positive:* $++++$ (4) or $+++$ (3) in the first tube. *Weakly positive:* $++$ (2) or $+$ (1) in the first tube. *Doubtful:* $\pm$ in the first tube. *Negative:* — in the first tube.

Slightly *anticomplementary reactions* may be safely reported as follows: $4 \pm =$ positive, $4\ 1 =$ positive, $4\ 2 =$ doubtful, $3\ 1 =$ doubtful, $3 \pm =$ doubtful, $3\ 3 =$ negative, $2\ 2 =$ negative, $2\ 1 =$ negative, $1\ 1 =$ negative, $1 \pm =$ negative, $\pm \pm =$ negative.

With serums heavily contaminated with bacteria and those which are chylous or heavily tinged with hemoglobin from spontaneous hemolysis in which the presence of thermostabile anticomplementary substances is suspected, a modified Sachs method is recommended for their preparation as follows:

1. Heat 0.5 c.c. of serum at 55° to 56° C. in a water bath for 30 minutes.

2. Add 4.1 c.c. of accurately titrated N/300 hydrochloric acid and mix.

3. After standing one-half hour at room temperature, centrifuge thoroughly and discard the sediment.

4. To the supernatant fluid add 0.4 c.c. of 10 per cent sodium chloride solution. The acid is fixed by the precipitate of globulin, hence neutralization is unnecessary.

5. This gives a 1:10 dilution of original serum ready for testing.

6. Place 1 c.c. in each of two tubes and proceed as above described. In these

tests the dose of diluted serum is equivalent to 0.1 instead of 0.2 c.c. of undiluted serum. The reactions, however, are recorded and reported exacty as above described.

EAGLE MODIFICATION OF THE WASSERMANN TEST

Preparation of Antigen.—If a uniform preparation of dried powdered beef heart is not available. the technician may prepare his own. The heart tissue must be quite fresh, and the fat, pericardium, and the blood vessels should be removed. The muscle is ground in an ordinary meat grinder, and then dried by the addition of 150 c.c. of acetone to each 100 grams of tissue. After about an hour at room temperature, with frequent shaking, the acetone is removed·by filtration, the tissue is shaken with a second portion of acetone (150 e.c.) for a few minutes, and the mixture again filtered. The acetone filtrates are discarded. The tissue is spread in a thin layer on a clean surface and thoroughly dried in a 37° C. incubator for 24 hours, when it is turned and dried again for 24 hours. The dry sheet is now pulverized as finely as possible in a mortar, or preferably. in a pulverizing machine.

(a) *Preparation of the tissue extract.*—50 grams of the dried powdered beef heart. preferably a pooled pieparation such as those available commercially (e.g.. Difco). are extracted with 250 c.c. of pure anesthesia ether (5 e.c. per gram powder) for 15 minutes at 30° to 37° C.. with frequent shaking. The mixture is filtered with suction, and the extraction is repeated with fiesh ether for a total of 4 extractions. All the ether extracts are discarded. After the fourth filtration, the beef heart powder is washed on the filter with 100 c.c. of fresh ether, dried, and then extracted for 5 days with 250 c.c. of absolute alcohol (5 c.c. per gram powder). The alcohol extract is filtered and the moist powder is washed on the filter with small portions of fresh alcohol until the volume of the combined alcoholic filtrate and washings is equal to 250 c.c. (5 c.c. per gram powder). The clear, straw-yellow extract is the basic antigen, and contains approximately 1.2 to 1.5 per cent solids. It is now fortified with cholesterol which is added to a concentration of 0.6 per cent (6 mg. per c.c. extract). The cholesterol is dissolved by boiling. This completed antigen, tightly stoppered, retains its reactivity almost indefinitely (moie than 8 years) at room temperature. For use in the test, it is diluted fresh each day by slowly pouring from 80 to 200 volumes of 0.85 per cent salt solution into 1 volume of the antigen. The exact quantity of salt solution to be used is determined once for each lot of antigen by the technic to be described. One cubic centimeter of antigen suffices for approximately 100 tests.

It is to be noted that the same basic extract, fortified with 0.6 per cent cholesterol and 0.6 per cent corn germ sterol, is used in the Eagle flocculation test.

(b) *Anticomplementary and hemolytic titration of antigen.*—Each lot of antigen should be tested once for its anticomplementary and hemolytic activity to ensure that these undesirable properties are not so pronounced as to interfere with its use in the test. The technic of these titrations is given in Tables I and II. Antigens prepared by the method just described are usually not anticomplementary in more than a 1·6 dilution and aie not significantly more hemolytic than pure alcohol.

The use of a 1:120 dilution in the test proper (vide infra) thus provides a wide margin of safety.

TABLE I

HEMOLYTIC TITRATION OF ANTIGEN

	Antigen dilution *						
	1	1:2	1:3	1:4	1:6	1:8	1:12
Antigen dilution, c.c.	0.4	0.4	0.4	0.4	.01	0.4	0.4
0.85 per cent salt solution, c.c.	.8	.8	.8	.8	.8	.8	.8
Cell suspension, c.c. †	.8	.8	.8	.8	.8	.8	.8

Hemolysis is Read After ½ Hour at 37° C.

Example of reading of hemolysis.	Complete	None	None	None	None	None	None

Conclusion: The antigen is not significantly hemolytic.

* These dilutions are readily prepared by the following procedure:

Whole antigen, c.c.	0.4	0.2	0.13	0.1	0.07	0.05	0.035
Salt solution, c.c.	0	.2	.27	.3	.33	.35	.37
Final dilution of antigen	1	1:2	1:3	1:4	1:6	1:8	1:12

† Can be sensitized or unsensitized, but should be 1.5 per cent by volume.

TABLE II

ANTICOMPLEMENTARY TITRATION OF ANTIGEN

	Antigen dilution *						
	1	1:2	1:3	1:4	1:6	1:8	1:12
Antigen dilution, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.85 per cent salt solution, c.c.	.4	.4	.4	.4	.4	.4	.4
Complement, 1:10, c.c.	.4	.4	.4	.4	.4	.4	.4

After 4 hours at 0° to 5° C., followed by ½ hour at 37° C., add 0.8 c.c. of sensitized cells to all the tubes

Example of reading of hemolysis after ½ hour at 37° C.	Complete	0	0	Partial	Complete	Complete	Complete

Conclusion: Antigen is anticomplementary up to 1:4 dilution.

* These dilutions are readily prepared by the following procedure:

Whole antigen, c.c.	0.4	0.2	0.13	0.1	0.07	0.05	0.035
Salt solution, c.c.	0	.2	.27	.3	.33	.35	.37
Final dilution of antigen.	1	1:2	1:3	1:4	1:6	1:8	1:12

(c) *Determination of the optimum antigen dilution.*—As in the case of the anti-complementary titration, the optimum dilution of the antigen need be determined only once with each lot of antigen. The importance of this titration is not generally realized. A typical titration illustrating the method is given in Table III. In

this example, the most sensitive, and thus the optimum dilution, is approximately 1:140. If the powdered beef heart used for the antigen is a pooled, uniform product and if the method of preparation is adhered to rigidly, it is generally found that the optimum antigen dilution is also quite uniform, not deviating significantly from the 1:120 to 1:160 value of the following titration.

TABLE III

DETERMINATION OF THE OPTIMUM ANTIGEN DILUTION

Antigen dilution *	Strongly positive serum	Result of Wassermann test on the same serum diluted with salt solution						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
1:40	+	+	+	±	0	0	0	0
1:80	+	+	+	+	±	0	0	0
1:100 ...	+	+	+	+	+	0	0	0
1:120 ...	+	+	+	+	+	±	0	0
1:160 ...	+	+	+	+	+	±	0	0
1:200 ...	+	+	+	+	+	0	0	0

+ = positive (no hemolysis).
± = doubtful (partial hemolysis).
0 = negative (complete hemolysis).
* The antigen dilutions to be used in the above-mentioned titration can be prepared as follows:

1:40 antigen dilution c.c...	8.0	4.0	3.2	2.7	2.0	1.6
Salt solution, c.c.	0	4.0	4.8	5.3	6.0	6.4
Final dilution of antigen..	1:40	1:80	1:100	1:120	1:160	1:200

Similarly, the serum dilutions can be readily prepared as follows:

Serum, c.c.	4.0	2.0	1.0	0.5	0.25	0.125	0.062
Diluent, c.c.	0	2.0	3.0	3.5	3.75	3.9	4.0
Final dilution of serum...		1:2	1:4	1:8	1:16	1:32	1:64

In preparing the antigen for daily use in the test, the correct volume of salt solution is slowly poured into one volume of antigen. The antigen dilution should be opalescent and homogeneous, containing no visible granules.

Preparation of Complement.—At least five, and preferably more, guinea pigs are bled from the heart; the clots are broken up with a glass rod and centrifuged. Bleeding large pigs (more than 1 pound in weight) for comparatively small quantities (5 to 10 c.c.) at intervals of 4 to 6 weeks, is more economical than exsanguinating the animal.

The small laboratory which cannot pool the fresh serum of several pigs for each series, or which cannot obtain such pooled fresh serum from a central source of supply, may have recourse to three methods of preservation. (1) Multiple containers of complement serum may be vacuum-dried from the frozen state and vacuum-sealed on the Flosdorf-Mudd apparatus. Such complement remains fully active for at least 10 months if stored in the refrigerator. (2) Although complement retains its activity, when frozen at very low temperature ($< 20°$ C.), over a period of weeks and even months, such freezing is not feasible in the ordinary laboratory. (3) Complement may be preserved by the addition of salt. Numerous methods of "salting" complement have been recommended. As simple and as satisfactory as any is the addition of sodium chloride in bulk to the complement

serum (80 mg. of NaCl per c.c. serum, dissolved by shaking). For actual use in the test, the salted complement is diluted with 9 volumes of water (e.g., 1 c.c. complement + 9 c.c. distilled water) to make a 1:10 dilution. ·

Perhaps the most satisfactory method of preserving complement in the small laboratory is a combination of (2) and (3)—the container of salted complement is placed in the freezing compartment of a mechanical refrigerator. So stored, it undergoes no demonstrable deterioration in 2 weeks.

The Preparation of the Serum or Spinal Fluid to Be Tested.—The coagulated blood is separated from the sides of the tube with a clean glass rod and centrifuged for 5 to 10 minutes at 1,000 to 1,500 r.p.m. 1 c.c. of the clear serum is pipetted into a numbered tube, which is then placed in a water bath at 56° C. for 15 to 20 minutes. An incompletely coagulated specimen, or one prevented from coagulating by the addition of oxalate or citrate (neither of which should be used in the Wassermann reaction), will develop a precipitate of coagulated fibrinogen upon inactivation. Contrary to published reports, even large amounts of hemoglobin dissolved in the serum do not per se affect the result save so far as they obscure the reading of hemolysis. Similarly, the only objection to blood older than 24 hours is the fact that it tends to become anticomplementary with age, particularly if conditions facilitate multiplication of bacteria. Whenever feasible, the serum should be inactivated immediately before testing. If it is inactivated the day before the actual test, it should be reheated for 5 minutes at 56° C. before proceeding with the test on the following day.

Native amboceptor for sheep cells may be removed from the inactivated serum by adding 0.1 c.c. of a thick (20 to 40 per cent) suspension of washed sheep cells to each cubic centimeter of inactivated serum. The serum and cells are thoroughly mixed and centrifuged 5 minutes later in order to remove the cells. An alternative method is to add the thick sheep cell suspension to the whole cold blood, mix thoroughly with a clean rod, and centrifuge after 15 minutes in the refrigerator. The latter procedure has the advantage of simplicity for it eliminates the necessity of a double centrifugation for each serum; it has the disadvantage of causing slight hemolysis.

The removal of native amboceptor unquestionably results in an increased sensitivity. Nevertheless, it is perhaps an unnecessary complication if a flocculation test is carried out in parallel with the Wassermann. The flocculation test ensures the detection of practically all serums which might be Wassermann negative because of their native amboceptor content.

Spinal fluid contains neither complement nor amboceptor in significant quantities, and therefore requires neither inactivation nor the absorption of native amboceptor. The whole fresh fluid is used as such.

Salt Solution.—The salt solution used in diluting antigen, complement, serum, cells and amboceptor is a 0.85 per cent solution of C. P. NaCl in distilled water. It is advisable but not absolutely necessary to add a trace of alkaline buffer, as old distilled water may be sufficiently acid because of dissolved CO_2 to accelerate the deterioration of dilute complement. It is convenient to keep a stock solution

of 17 per cent NaCl. Fifty c.c. of this is diluted with 950 c.c. of distilled water to form the 0.85 per cent solution used in the test.

Routine Qualitative Serum Test.—Three tubes are advisable although only two are essential. Tube 1 is the serum control. containing serum and complement, but salt solution instead of antigen.

	Serum control	Test proper	
Whole serum, c.c.	0.2	0.2	0.1
Complement. 1:10, c.c.	.4	.4	.4
Antigen dilution, c.c.	0	.4	.4
0.85 per cent NaCl, c.c...........	.6	*.2	*.3

* May be omitted.

Tubes 2 and 3 are the test proper. each containing 0.4 c.c. complement, 0.4 antigen, and different quantities of serum (0.2 and 0.1 c.c.). The addition of salt solution to tubes 2 and 3 is not essential but serves to bring the total volume in all three tubes up to 1.2 c.c.. corresponding to 0.4 c.c. of each of the three reagents.

One antigen control suffices for the entire series of tests, and two complement controls are also set up, as here indicated.

	Antigen control	Complement controls (in duplicate)				
Antigen dilution, c.c.	0.4	0.4	0.4	0.4	0.4	0.4
Complement, 1:10, c.c.	.4	.4	.2	.13	.1	0
Salt solution, c.c.	.4	.4	.6	.7	.7	.8

Quantitative Serum Test.—If a quantitative determination of the reagin content of a known positive serum is desired, a series of serum dilutions may be prepared by placing 0.4 c.c. of salt solution in each of a series of tubes. To the first tube is added 0.4 c.c. of serum, and 0.4 c.c. of the resulting mixture is transferred to the following tube: 0.4 c.c. is withdrawn from tube 2 and transferred to tube 3, and the process is repeated with all the tubes of the series. The final set-up is then as follows:

Serum control	Test proper						
Serum, c.c., 0.2	0.2	0.1	0.05	0.025	0.0125	0 0062	0.031
	in a total volume of 0.4 c.c. corresponding to a dilution of						
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256

It is a technically simpler procedure to prepare a single 1:20 dilution of the serum (0.1 c.c. + 1.9 c.c. salt solution) and distribute the serum as follows:

Whole serum, c.c.	0.2	0.05				
Serum, 1:20, c.c.			0.4	0.2	0.1	0.05
Salt solution, c.c.	.2	.35	0.	.2	.3	.35

The dilution so obtained are 1:2, 1:8, 1:20, 1:40, 1:80, 1:160, in a volume of 0.4 c.c. This is the recommended procedure. Antigen and complement are then added exactly as in the routine test (0.4 c.c. of each dilution).

Spinal Fluid Test.—The routine spinal fluid Wassermann is a quantitative test. Except for the quantities used and the fact that the fluid is not inactivated, the set-up of the test is similar to that of the quantitative serum test:

	Spinal fluid control	Test proper						
Spinal fluid, c.c.	.2	1.0	0.6	0.4	0.2	0.1	0.05	0.025
Complement, 1:10, c.c.	*.2	.2	.2	.2	.2	.2	.2	.2
Antigen dilution, c.c.	0.	.2	.2	.2	.2	.2	.2	.2

*The quantities of the reagents are halved in order to conserve spinal fluid. It is to be noted that the maximum amount of spinal fluid used relative to the other reagents is 10 times that used in the serum tests.

The entire series, serum and spinal fluid tests and controls alike, are then placed in the ice box for 3 to 4 hours.

While the tests are in the ice box, the sensitized cell suspension is prepared and tested.

Preparation of the Sensitized Cell Suspension.—Because this technic calls for the use of a fixed quantity of a pooled, standard complement, it becomes possible to set up the diagnostic tests as the first step in the daily performance of the Wassermann reaction, to place these tests in the refrigerator for their primary incubation of 3 to 4 hours, and then proceed to the preparation of the sensitized cell suspension. The cells can be washed, the amboceptor titrated, and the titration checked within 2 hours. This leaves ample time for other routine work before *the tests are placed at 37° C. for their secondary incubation of 30 minutes* prior to the addition of the cells.

(a) *Preparation of the stock 3 per cent suspension of sheep cells.*—Citrated sheep blood (1 part of 5 per cent citrate and 5 to 10 parts of blood) or defibrinated blood is collected as aseptically as conditions permit. The method of choice is to bleed the animals from the jugular vein into a sterile vessel containing the citrate. The addition of sucrose in bulk to a concentration of 2.5 per cent (2.5 grams per 100 c.c. blood) serves to postpone their spontaneous disintegration. Sterile sheep blood generally remains serviceable for 1 to 3 weeks if stored at 0° to 5° C.

For use in the test, 1 volume of blood is washed with 10 to 15 volumes of 0.85 per cent salt solution, the mixture is centrifuged, and the supernatant fluid carefully withdrawn. The sedimented cells are resuspended in a second portion of salt solution and again centrifuged, this time in a graduated tube, until the volume of sedimented cells becomes constant. Ten to 15 minutes at 2,000 to 2,500 revolutions per minute suffice. The supernatant fluid is carefully withdrawn, and the measured cell sediment is resuspended in 32 volumes of salt solution to form the stock 3 per cent suspension of unsensitized cells. This must be prepared fresh daily.

(b) *Titration of amboceptor.*—The minimal hemolytic quantity (unit) of amboceptor must be determined daily for the particular cell suspension to be used that day, by the technic of Table IV. This hemolytic unit of amboceptor should be a 1:2,400 dilution or higher; and an amboceptor should be discarded if it is so inactive that the hemolytic unit represents, e.g., a 1:1.000 dilution.

The unit of amboceptor in the example cited is a 1:3.000 to 1:4,000 dilution, or, interpolating. approximately a 1:3,500 dilution. An amboceptor dilution is now prepared containing 2½ units (in the example cited, a 1:1,400 dilution). This amboceptor dilution is poured into an equal volume of the 3 per cent cell suspension to form the 1½ per cent suspension of sensitized cells which is actually used in the test.

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TABLE IV

TECHNIC OF AMBOCEPTOR TITRATION

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	Amboceptor titration *						
	1:1000	1:1500	1:2000	1:3000	1:4000	1:6000	1:8000
Amboceptor dilution, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
3 per cent cell suspension, c.c.	.4	.4		.4	.4	.4	.4
Salt solution, c.c.	.8	.8	.8	.8	.8	.8	.8
Complement, 1:10, c.c.	.4	.4	.4	.4	.4	.4	.4
Example of reading of hemolysis after ½ hour at 37° C.	Complete	Complete	Complete	Complete	Partial	Partial	None

* The following is a simple method of setting up these amboceptor dilutions:

1:1000 amboceptor, c.c.	0.4	0.27	0.2	0.13	0.10	0.067	0.05
Salt solution, c.c.	0.0	0.13	0.2	0.27	0.30	0.34	0.35
Final dilution of amboceptor....	1:1000	1:1500	1:2000	1:3000	1:4000	1:6000	1:8000

Check on the amboceptor titration.—The sensitization of the cells should be completed within an hour. One set of complement controls previously placed in the refrigerator along with the tests, and containing 0.4, 0.2, 0.13, and 0.1 c.c. of complement in a total volume of 1.2 c.c., is now removed from the refrigerator, and 0.8 c.c. of the sensitized cell suspension is added to each tube. The degree of hemolysis is read after 30 minutes at 37° C. If the amboceptor titration was correct, the 0.4 c.c. of 1:10 complement used in the test represents 2 to 2½ times the amount necessary to cause complete hemolysis in 30 minutes. Accordingly, the first two tubes of the complement titration should be completely hemolyzed; tube 3 should be partially hemolyzed, and tube 4 should show little or no lysis. Any error in the amboceptor titration becomes immediately apparent. If only the first tube is completely hemolyzed, the cells have been inadequately sensitized and more amboceptor should be added. If three tubes show complete lysis, an excess of amboceptor has been used. The technician must reconcile himself to a sudden decrease in the sensitivity of the test, or a second portion of blood must be washed and sensitized with the correct amount of amboceptor, somewhat less than that used in the first lot.

Secondary Incubation of the Tests at 37° C., and the Addition of the **Sensitized Cells.**—After 3 to 4 hours at 0° to 5° C., the tests and controls are placed in the 37° C. water bath for 30 minutes. Eight-tenths (0.8) c.c. of the sensitized cell suspension is then added to all the tubes, which are vigorously shaken and replaced at 37° for their final incubation of 20 to 30 minutes.

This leeway of 10 minutes is allowed the technician to compensate for any slight error in the sensitization of the cells as detected by the complement check just described. If tube 2 of the complement control, which contains half the amount of complement used in the test, is slow to hemolyze, requiring, for example, 30 minutes for complete lysis, then the tests also are given 30 minutes. If, on the other hand, tube 2 shows complete hemolysis in 20 minutes, the results of the tests also should be read in 20 minutes after the addition of the sensitized cells. The futility of any stop watch precision is apparent when we remember that it requires 5 to 15 minutes merely to add cells to a large series of tests.

Reading of Results.—(a) The *antigen controls* should be completely hemolyzed. Properly diluted, the antigen fortified with 0.6 or 1 per cent cholesterol is not demonstrably anticomplementary under the conditions of the test. Failure to hemolyze indicates that the complement is defective and is deteriorating under the conditions of the test.

(b) The *complement controls* incubated along with the tests should show approximately the same degree of hemolysis as the first set used to check the amboceptor titration. Any pronounced differences indicate either that the complement is defective and is deteriorating under the conditions of the test, or that the antigen is significantly anticomplementary under the conditions of the test. Such deterioration with fresh complement indicates that the guinea pigs are in poor condition, and necessitates the greatest caution in the reading of results.

(c) *Reading of tests proper.*—If the serum as such destroys complement, in the absence of antigen, it is anticomplementary—a result which offers no evidence as to the presence or absence of syphilitic infection. If the serum control is completely hemolyzed, and the tubes of the test containing serum, complement, and antigen show no hemolysis, complement has been fixed by a lipoid-reagin compound, and the result is positive. If all three tubes show complete hemolysis, the complement has not been fixed and the result is negative. Finally, if the tests show partial hemolysis, the serum control being competely hemolyzed, only part of the complement has been fixed, and the result is doubtful.

In the quantitative Wassermann test the result is given as the maximum dilution of serum which continues to give a positive result. Similarly, in the spinal fluid test, a Wassermann positive fluid may be said to be positive down to, for example, 0.5 c.c.

METHOD FOR THE DETERMINATION OF VITAMIN C IN URINE

Principle.—This is based upon the reduction of Folin's phospho-tungstic acid reagent by ascorbic acid. It offers the advantages of simplicity, speed and accuracy. The method is that of Medes modified by Shrader.

Reagents.—1. *M. formaldehyde:* 8 c.c. of formalin (app. 36%) diluted to 100 c.c.

2. *Combined buffer:* 273 gms. sodium acetate (crystalline) plus 34.3 c.c. of glacial acetic acid, diluted to 1 liter.

3. *Folin's phospho-tungstic acid reagent:* Transfer 100 gms. of sodium tungstate (free from molybdate) to a 500 c.c. Florence flask. Mix 32 to 33 c.c. of 85% phosphoric acid with 150 c.c. of water. Pour the resulting solution on to the tungstate and mix. Add a few pebbles and boil *very gently* over a micro-burner for 1 hour. Loss of liquid during the boiling is prevented by using, as a condenser, a funnel holding a 200 c.c. flask filled with cold water. At the end of the boiling period decolorize with a little bromine water, boil off the excess bromine. cool, and dilute to 500 c.c.

4. *Ascorbic Acid Standard:* Weigh *accurately* in the neighborhood of 15 mg. of ascorbic acid and place in a dry 100 c.c. volumetric flask. Then weigh approximately 1 gm. of sodium bisulphite into a beaker, add 1 c.c. of acetate buffer and about 50 c.c. of water. When the bisulphite has dissolved, add to the ascorbic acid and dilute to 100 c.c. The bisulphite is added to prevent oxidation of the ascorbic acid.

Procedure.—To 5 (1-5) c.c. of urine in a 25 c.c. volumetric flask add 1 c.c. of formaldehyde solution. Into three other 25 c.c. volumetric flasks pipette 1, 2 and 4 c.c. of the ascorbic acid standard respectively. To each flask add 5 c.c. of the combined buffer solution followed by 1 c.c. of the uric acid reagent. Adjust the volume and read in the colorimeter after 20 minutes.

Calculation.—$C_s \times \dfrac{RS}{RU} \times \dfrac{\text{24-hour output}}{\text{Vol. Urine used in test}}$ = mg. of ascorbic acid in daily output

C_s is the concentration of the standard used, RS is the reading of the standard, and RU is the reading of the unknown.

Comments.—In order to remove any interference caused by the color of the urine, on the blue color developed in this method. a light filter consisting of a 2 per cent solution of potassium iodide containing enough iodine to give a decided red color has been successfully used.

If a cloudiness develops in the unknown, it may be centrifuged and the supernatant liquid used for comparison.

MICROMETHOD FOR THE DETERMINATION OF VITAMIN C IN PLASMA
(FARMER AND ABT)

Reagents.—1. *The Dye:* Sodium-2, 6-dichlorobenzenone indophenol may be obtained in powder form from the Eastman Kodak Company. In order to secure a clear solution of the proper accuracy for good work, Farmer and Abt recommend the use of this powder rather than tablets which are also commercially available. The powder is extracted with boiling water to make a stock solution.

which must then be diluted and standardized against pure crystalline ascorbic acid. The dilute stock solution is further diluted (1:2) for titrating the deproteinized plasma. It is stated that the dye varies in appearance; lots having a dark green color are preferred, while a dark purple color is less desirable, the material being harder to dissolve.

2. *Metaphosphoric Acid.* (HPO_3): This material is used as the deproteinizing agent for the plasma in a strength of 5%. It is also used as a solvent for ascorbic acid in the standardization of the stock dye in a strength of 2.5 per cent. In solutions of HPO_3, the dye assumes a pink color instead of the deep blue seen in neutral solutions.

3. *Ascorbic Acid:* Any pure crystalline ascorbic acid may be used to standardize the dye. For greatest accuracy, it is recommended that crystals sealed in vacuum ampules be used.

4. *Lithium Oxalate:* This material is preferred as an anticoagulant to other oxalates. A 1-mm. pile of the powder on the end of a toothpick is placed in the micro-blood bottle.

Apparatus.—In addition to the ordinary glassware which is a part of the equipment of any chemical laboratory, are required a microburette, a chemical balance and a centrifuge. Special apparatus which is required consists of the micromethod bottles and a special microburette reading directly to 0.002 c.c. The special microburette is an accurately graduated capillary pipette held horizontally in a cast fixture. Over the blunt end there is slipped a piece of medium-wall rubber tubing, the other end of which is sealed with a glass plug. This tubing is so placed that it may be compressed by a clamp having a finely threaded screw. When the whole burette is filled with mercury, and all air expelled, turning the screw accurately controls the mercury. The mercury acts as a fluid piston and the dye may be drawn in or pushed out by its action. This apparatus is obtainable from E. H. Sargent & Co.

Procedure.—The first step in the procedure is the standardization of the dye. After diluting the stock dye 1:10 with water, this is titrated against an accurately made solution of ascorbic acid in 2.5% HPO_3. The calculations are as follows: Suppose exactly 59.2 milligrams of ascorbic acid were weighed out and made up to 100 c.c. If this solution is diluted 1:10, each c.c. of the dilute ascorbic acid solution contains 0.0592 milligrams of ascorbic acid. Suppose 2.27 c.c. of dilute dye produce the first faint pink color in 2 c.c. of the dilute ascorbic acid solution. Suppose 0.10 c.c. of dilute dye must be added to the HPO, blank to produce the same pink color. Then:

$$(2.27 - 0.10 \text{ c.c.}) = (2 \times 0.0592) \text{ mg.}$$

$$\text{or, each c.c. of dye} = \frac{2 \times 0.0592}{2.17} \text{ milligrams of ascorbic acid}$$

$$= 0.0545 \text{ mg. (standardization factor)}$$

The next step is to obtain the blood, centrifuge out the cellular elements, reproteinize, and titrate. Several of these operations require, in the calculations, cor-

rective factors in order to express the final result as milligrams of ascorbic acid per 100 c.c. of blood. Each factor is dealt with separately below:

(a) 0.1 c.c. of plasma is taken. Since this is to represent 100 c.c. of blood, a factor of 1000 must be introduced.

(b) Of the 0.4 c.c. of diluted deproteinized plasma, only 0.2 c.c. are used for the titration. This introduces a factor of 2.

(c) The dilute standard dye is further diluted for the micro-titration, introducing a factor of ½.

Multiplying all these factors together gives:

$$1000 \times 2 \times \tfrac{1}{2} = 1000$$

Suppose 0.026 c.c. of dye is used in the plasma titration, and suppose that 0.002 c.c. of this same dye is needed to give the HPO₃ blank the same pink color. Subtracting the latter from the former gives 0.024 c.c. This multiplied by the standardization factor, multiplied by the factor 1000, gives the finished result expressed in milligrams per 1000 c.c. of blood, as follows:

$$0.024 \times 0.0545 \text{ (standardization factor)} \times 1000 = 1.308 \text{ mg./100 c.c.}$$

Interpretation.—The normal range of vitamin C (ascorbic acid) in the blood by this method has been established as 0.7 to 1.2 milligrams per 100 c.c. Values which are below 0.5 milligram are considered to indicate a marked insufficiency of vitamin C intake.

METHOD FOR THE DETERMINATION OF SULFANILAMIDE IN THE BLOOD AND URINE

(BRATTON AND MARSHALL)

Principle.—This method for the determination of free sulfanilamide in filtrates of the blood, urine and various body fluids, can be used also for sulfapyridine, sulfathiazole or any derivative of sulfanilamide in which the amino group is free or can be liberated by hydrolysis. The reaction depends upon the presence of an amino group substituted in the benzene ring. The method is based on the diazotization of the para-aminobenzene-sulfonamide with nitrous acid, destroying the excess nitrous acid, and then coupling the resulting diazo compound with N-(1-naphthyl) ethylenediamine dihydrochloride which can be obtained from the LaMotte Chemical Company. A purplish red azo-dye is produced which can be readily estimated colorimetrically. The color reaction is extremely sensitive so that the drug can be detected in a dilution of 1 part per million or more.

Reagents.—These should be kept at refrigerator temperature to avoid decomposition.

1. *Trichloracetic acid solution,* containing 15 gm. dissolved in water and diluted to 100 c.c.

2. *Sodium nitrite* solution prepared weekly by dissolving 0.1 gm. in 100 c.c. of water.

3. Aqueous solution of *N-(1-naphthyl) ethylenediamine dihydrochloride* (Eastman Kodak No. 4836 or LaMotte Chemical Company) prepared by dissolving 100 mgm. in 100 c.c. of water and keeping in a dark colored bottle.

4. *Saponin solution* prepared by dissolving 0.5 gm. in 1000 c.c. of water.

5. *Ammonium sulfanate solution* prepared by dissolving 0.5 gm. in 100 c.c. of water.

6. 4 N hydrochloric acid.

7. *Stock standard sulfanilamide solution* prepared by dissolving 100 mg. in hot water. Cool and dilute to 500 c.c. (5 c.c. = 1 mg.). This will keep several months in a refrigerator.

8. *Dilute standard sulfanilamide solutions* are prepared as follows:

	Concentration (mg. per 10 c.c.)	Stock Standard	15% Trichloracetic acid	Water q.s. to:
No. 1	0.02	1.0 c.c.	18 c.c.	100 c.c.
No. 2	0.05	2.5 c.c.	18 c.c.	100 c.c.
No. 3	0.10	5.0 c.c.	18 c.c.	100 c.c.

Since various sulfanilamides may give varying amounts of color it is advisable to use that being administered.

Procedure for Free Sulfanilamide.—To 2 c.c. of blood add 30 c.c. of saponin solution and allow to lake completely. Add 8 c.c. of trichloracetic acid solution, mix and filter. To 10 c.c. of the filtrate add 1 c.c. of the sodium nitrite solution and let stand 3 minutes. Add 1 c.c. of the ammonium sulfamate solution and let stand 2 minutes. Add 1 c.c. of the N-(1-naphthyl) ethylenediamine dihydrochloride reagent; mix and read any time within an hour. Compare in the colorimeter with the nearest standard:

Calculation.—Set the unknown at 10:

$$\text{Standard No. 1} \ldots\ldots \text{mg./100 c.c.} = \frac{S}{10} \times 0.02 \times 2 \times 100 = 0.4 \text{ S}$$

$$\text{Standard No. 2} \ldots\ldots \text{mg./100 c.c.} = \frac{S}{10} \times 0.05 \times 2 \times 100 = S$$

$$\text{Standard No. 3} \ldots\ldots \text{mg./100 c.c.} = \frac{S}{10} \times 0.1 \times 2 \times 100 = 2.0 \text{ S}$$

Procedure for Total Sulfanilamide.—To 10 c.c. of filtrate add 0.5 c.c. of 4 N hydrochloric acid. Heat in boiling water bath for 1 hour and cool. Adjust volume to 10 c.c. and proceed as for the determination of free sulfanilamide.

The *conjugated sulfanilamide* is obtained by subtracting the free from the total.

Procedure for Sulfanilamide in the Urine.—Protein-free urine is diluted with water to contain about 1 to 2 mg. per cent of sulfanilamide. 50 c.c. of diluted urine plus 5 c.c. of 4 N hydrochloric acid are diluted to 100 c.c. 10 c.c. of this second dilution are treated in the same manner as blood filtrate for the determina-

tion of free sulfanilamide. For the determination of total sulfanilamide, 10 c.c. are heated without the further addition of acid. Urine containing protein is examined in the same manner as blood.

METHOD FOR THE DETERMINATION OF SULFATHIAZOLE IN THE BLOOD

The procedure for determination of *free sulfathiazole* in the blood is as follows: Dilute 2 c.c. of oxalated blood with 30 c.c. of saponin solution. After complete laking (1 or 2 minutes), add, with shaking, 8 c.c. of the trichloracetic acid solution, thus making 40 c.c. of a 1:20 dilution of blood. Allow to stand five minutes or more and filter. (If only a smaller volume of blood is available, use proportionately less of the other constituents.)

The free sulfathiazole is determined in the filtrate as follows: 1 c.c. of the sodium nitrite solution is added to 10 c.c. of the filtrate. After three minutes' standing, 1 c.c. of the ammonium sulfamate solution is added, and after two minutes' further standing, 1 c.c. of the solution of N-(1-naphthyl) ethylenediamine dihydrochloride is added. The color of this unknown is compared with an appropriate standard which has been treated identically with the solutions of sodium nitrite, ammonium sulfamate, and N-(1-naphthyl) ethylenediamine dihydrochloride. The color develops at once after the addition of the last reagent and does not change for an hour.

To determine the *total sulfathiazole* (free and conjugated), 10 c.c. of the filtrate should be treated with 0.5 c.c. of 4 N hydrochloric acid, heated in a boiling water bath for one hour, cooled, and the volume adjusted to 10 c.c. For this purpose it is best to use Pyrex test tubes with a constriction on which the calibration mark for 10 c.c. is etched. The subsequent procedure is the same as for determination of free sulfathiazole.

In making sulfathiazole determinations by the suggested procedure, if the level is found to be from 1 to 10 mg. per cent, a correction of 15 to 20 per cent should be added when the blood dilution of 1:20 has been used. When dilutions of blood of 1:50 or 1:100 are employed, the recovery is quantitative with larger amounts than 5 mg. per cent and no correction is needed. Such greater dilutions are to be obtained by using additional amounts of saponin solution.

METHOD FOR THE DETERMINATION OF SULFAPYRIDINE IN THE BLOOD

The following bedside method by Ratish and Bullowa is a modification of the Marshall test which can be conducted by the physician or technician at the bedside in ten minutes and allows time for simultaneous duplicate tests.

Reagents.—1. Ether.
2. 15 per cent trichloracetic acid solution.
3. 0.1 per cent solution of sodium nitrite.

    4. 1 per cent solution of urea.

    5. A solution containing 1 c.c. of a-dimethylnaphthylamine in 250 c.c. of 95 per cent ethyl alcohol. (Keep in a dark dropping bottle.)

Apparatus.—1. 2-c.c. Luer syringe and needle.

    2. 1 test tube of 20-c.c. capacity with round bottom, stoppered with cork and marked at 1 c.c. and 6 c.c. 1 centrifuge tube with long taper, graduated to 15 c.c. in 0.1-c.c. divisions, fitted with rubber stopper.

    3. 5 dropping bottles to contain the reagents listed above.

    4. A comparator block 2.5 inches by 4.5 inches, with 4 openings to take test tubes 100 by 12 mm., and color standards.

Color Standards.—Dissolve 0.0075 gm. of phenol red in 100 c.c. of distilled water. To a series of five tubes, add 3.9 c.c. of fifteenth molar potassium dihydrogen phosphate (KH_2PO_4) and 6.1 c.c. fifteenth molar secondary sodium phosphate (NA_2HPO_4). Then follow with 0.2 c.c. of normal sodium hydroxide (NaOH) and phenol red as indicated. The volume of the phenol red should increase progressively in the different test tubes:

Amount of Phenol Red	Amount of Sulfapyridine
0.22 c.c.	Concentrations up to 4 mg. per cent.
0.30 c.c.	Concentrations up to 7 mg. per cent (usual in blood of recovered pneumococcic pneumonia patients).
0.46 c.c.	Concentrations up to 10 mg. per cent.
0.62 c.c.	Concentrations up to 12.5 mg. per cent (desirable in blood of meningitis patients).
0.86 c.c.	Concentrations up to 15 mg. per cent.

Phenol red solutions are transferred to 100 by 12-mm. test tubes and sealed. The value of each tube is attached and the standards protected from direct sunlight. New standards should be prepared every six months.

Procedure.—Into a Luer syringe draw approximately 1.5 c.c. of venous blood. Invert the syringe with the needle still attached and deliver the blood, drop by drop, into the round-bottomed test tube, to the 1-c.c. mark. From the other dropping bottle add ether to the 6-c.c. mark. Insert the stopper and shake vigorously for two minutes. The fluids will rapidly separate into two layers, with the ethereal extract of sulfapyridine in the upper layer. Slowly release the stopper. Cautiously decant the ethereal extract into the centrifuge tube to the 0.5 c.c. mark and set the extraction tube aside for duplicate tests.

By means of a dropping bottle add 15 per cent trichloracetic acid solution to the 5-c.c. mark, place the rubber stopper over the *mouth* of the tube, and shake vigorously for ten to twenty seconds. Add 0.5 c.c. of 0.1 per cent sodium nitrite solution (7 to 8 drops from a dropping bottle), bringing the solution to the 5.5-c.c.

mark. Again shake vigorously for twenty seconds, and add 0.5 c.c. of 1 per cent urea solution dropwise from a dropping bottle. Finally, add a-dimethylnaphthylamine to the 8.5-c.c. mark from its dropping bottle, close the tube with the rubber stopper, and invert once or twice. The white opalescence is soon replaced by a purplish-red color. After five minutes the color is fully developed and the tube may be compared with standards in the comparator block.

METHOD FOR THE DETERMINATION OF THIOCYANATES IN THE BLOOD

In the treatment of hypertension with thiocyanates concentrations of 6 to 12 mg. per 100 c.c. of blood give the maximum therapeutic results. Concentrations above 15 mg. per cent may produce toxic effects and concentrations above 35 mg. per cent may produce serious toxic manifestations. The method of Barker for their blood concentration is as follows:

Reagents.—1. 10 per cent *trichloracetic acid solution.*

2. *Ferric nitrate reagent.* Dissolve 50 gm. of crystallized ferric nitrate in 500 c.c. of distilled water. Add 25 c.c. of concentrated nitric acid and make up to 1 liter with distilled water.

3. *Thiocyanate standards:* Stock solution. Dissolve about 1 gm. of potassium thiocyanate in 800 c.c. of distilled water. Titrate a 20-c.c. portion of a standard silver nitrate solution (made by dissolving exactly 2.9195 gm. of silver nitrate in 1 liter of distilled water) acidified with 5 c.c. of concentrated nitric acid, with the potassium thiocyanate solution, using ferric ammonium sulfate as an indicator. Calculate the amount of water which it will be necessary to add to the potassium thiocyanate solution to make 20 c.c. equivalent to 20 c.c. of silver nitrate solution. Add the calculated amount of water, mix thoroughly and check the solution by another titration to make sure the potassium thiocyanate solution is exactly equivalent to the silver nitrate solution.

4. *Standard solutions.* Make three dilutions of the stock solution to give the following three standards: (1) 100 c.c. of stock diluted to 1 liter with water gives a standard which contains 0.5 mg. of the thiocyanate ion in 5 c.c. of solution. (2) 70 c.c. of stock diluted to 1 liter with water gives a standard which contains 0.35 mg. of the thiocyanate ion in 5 c.c. of solution. (3) 40 c.c. of stock diluted in 1 liter with water gives a standard which contains 0.2 mg. of the thiocyanate ion in 5 c.c. of solution.

Procedure.—Transfer 5 c.c. of the 10 per cent trichloracetic acid solution to a test tube. Add 5 c.c. of serum or plasma. Stopper and shake well. Allow to stand from ten to fifteen minutes. Filter through a small filter paper. The filtrate should be perfectly clear. If it is not, filter again through the same filter paper. Measure 5 c.c. of the filtrate into a clean, dry test tube. Add 1 c.c. of the ferric nitrate reagent; mix and read in a colorimeter with the standard solution set at 20 mm., choosing that standard which most nearly matches the unknown. The standards are made as follows: transfer 5 c.c. of each of the three standard solutions to three

test tubes. Add 5 c.c. of trichloracetic acid solution and 2 c.c. of the ferric nitrate reagent to each. Mix.

Calculation.—With the standard solution set at 20 mm. for the colorimetric comparison the calculation may be simplified to the three following forms, depending on the strength of the standard.

1. Using the 0.5 mg. standard, 200/reading-mg. of the thiocyanate ion in 100 c.c. of serum.

2. Using the 0.35 mg. standard, 140/reading-mg. of the thiocyanate ion in 100 c.c. of serum.

3. Using the 0.2 mg. standard, 80/reading-mg. of the thiocyanate ion in 100 c.c. of serum.

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